Aberrant regulation of the DNA replication licensing system and genomic instability in penile squamous cell carcinoma

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Declaration

I hereby declare that this thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, unless otherwise stated.

I further state that my thesis is not substantially the same as any I have submitted for a degree or diploma or other qualification at any other University. I declare that no part has already been or is being concurrently submitted for any such degree, diploma or other qualification.

Oliver Kayes

January 2014

Abstract

Penile cancer is a rare urogenital malignancy associated with an unusual geographical variability. Unifying hypotheses to explain this diversity include variations in human papilloma virus (HPV) infection and distinct socioeconomic influences. However, our understanding of the molecular drivers for penile carcinogenesis remains deficient. Furthermore, current prognostic and predictive tools for the clinical management of men with penile cancer have limited discriminatory roles. Radical surgery remains the cornerstone of treatment and effective adjuvant treatments are lacking. Despite improvements in surgical techniques and diagnostic methods, clinical outcomes appear to have plateaued in recent times. The DNA replication licensing factors are important cell cycle regulatory proteins which are showing promise as novel biomarkers and therapeutic targets in a broad range of tumour types. It may be possible to exploit the unique properties of the replication licensing system; as a relay station for upstream, signalling pathways, in order to develop utilitarian biomarkers and therapeutic targets for managing men with PeScc.

In this thesis, I show that there is aberrant regulation of the DNA replication licensing system in PeScc. Importantly, I demonstrate that the dysregulation of these molecules is associated with aggressive, genomically unstable tumour phenotypes, which in turn translates into important prognostic information with regards to patient survival. Furthermore, I have shown that multiparameter expression analysis of these proteins allows assessment of cell cycle kinetics in individual pathological specimens, parameters linked to the biological behaviour of these tumours. This novel form of cell cycle biomarker analysis may also be of value as a predictive test for assessing

the therapeutic effect of cell cycle phase specific anti-cancer agents or mechanistic drugs targeting the cell cycle machinery. Collectively, these studies highlight the prognostic, predictive and therapeutic utility of the DNA replication licensing system in PeScc.

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Kayes *et al* 2009. DNA replication licensing factors and aneuploidy are linked to tumour cell cycle state and clinical outcome in penile carcinoma. Clin Cancer Res. 2009 Dec 1;15(23):7335-44

Kayes *et al* 2007. Molecular and genetic pathways in penile cancer. Lancet Oncol. 2007 May;8(5):420-9

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List of Abbreviations

Ab	antibody
APC	anaphase promoting complex
Arg	arginine
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3 related
BAUS	British Association of Urological Surgeons
BC	basal compartment
BD	Bowen's disease
BP	Bowenoid papulosis
BSA	bovine serum albumin
BXO	balanitis xerotica obliterans
CNA	copy number alterations
Cdc	cell division cycle
CDK	cyclin dependent kinase
CDKi	cyclin dependent kinase inhibitor
CI	confidence interval
Cis	carcinoma in situ
COX	cyclo-oxygenase
CT	computed tomography
DAB	diaminobenzidine
DDK	Dbf4 dependent kinase

DNA	deoxyribonucleic acid
EAU	European Association of Urologists
EDTA	ethylenediaminetetra-acetate
EGTA	ethylene glycol tetraacetic acid
EQ	Erythroplasia of Queyrat
ESACP	European Society for Analytical Cellular Pathology
FACS	fluorescence activated cell sorting
FCM-DNA	DNA measured by flow cytometry
FITC	fluorescein isothiocyanate
H&E	haematoxylin & eosin
HCL	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	human papilloma virus
HR	hazard ratio
IARC	International Agency for Research on Cancer
ICM-DNA	DNA measured by image cytometry
IHC	immunohistochemistry
IOD	integrated optical density
KCl	potassium chloride
LI	labelling index
MAP	mitogen activated protein
MCM	minichromosome maintenance
MgCl2	magnesium chloride
MGLS	male genital lichen sclerosus
MMP	matrix metalloproteinases

MRI	magnetic resonance imaging
MSI	micro-satellite instability
mV	millivolt
NaCl	sodium chloride
NOS	squamous cell carcinoma of usual type
OR	odds ratio
ORC	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
Pap	Papanicolau
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PeIN	penile intraepithelial neoplasia
PeScc	penile squamous cell carcinoma
PG	prostaglandin
PI	prognostic index
PKMB	pseudoepitheliomatous, keratotic and micaceous balanitis
PMSF	phenylmethylsulfonyl fluoride
Pre-RC	pre-recognition complex
PWET	paraffin wax embedded tissue
Rb	retinoblastoma
RLF	replication licensing factor
ROC	receiver operating characteristics
RPM	revolutions per minute
RT	room temperature
SC	stem cell compartment

SCC	squamous cell carcinoma
Scc-Ag	squamous cell carcinoma antigen
SD	standard deviation
SDS	sodium dodecyl sulphate
SMI	small molecular inhibitor
SSL	secure sockets layer
TAC	transamplifying compartment
TBS	Tris buffered solution
Tris	tris[hydroxymethyl]amino-methane
Tween 20	polyoxyethylene (20) sorbitan monolaurate
UCL	University College London
UCLH	University College London Hospital
UK	United kingdom
USA	United States of America

Publications

Publications and presentations arising as a result of work conducted during the course of the study period relevant to this thesis:

Papers

Kayes O, Loddo M, Patel N, Patel P, Minhas S, Ambler G, Freeman A, Wollenschlaeger A, Ralph D, Stoeber K, Williams G
DNA replication licensing factors and aneuploidy are linked to tumour cell cycle state and clinical outcome in penile carcinoma. Clin Cancer Res. 2009 Dec 1;15(23):7335-44

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MRI as a an operative planning tool in the local-staging of penile cancer
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Kayes O, Durrant C, Floyd D, Withey S, Minhas S
Vertical rectus abdominis reconstruction (VRAM) in patients with advanced penile cancer.
BJU Int. 2006 Jan;99(1):37-41

Hegarty PK, **Kayes O**, Freeman A, Christopher N, Ralph DJ, Minhas S
A prospective study of 100 cases of penile cancer managed according to European Association of Urology guidelines.
BJU Int 2006 September;98(3):526-31

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What surgical resection margins are required to achieve oncological control in men with primary penile cancer?
BJU Int. 2005 Nov;96(7):1040-3

Abstracts

Academic Section of British Association of Urological Surgeons (BAUS) – London. Hunterian Lecture: "New biological horizons in penile cancer". 2010

24th Annual European Association of Urologists Congress (EAU) – Stockholm. "Replication licensing factors and aneuploidy predict outcome in penile cancer patients". 2009

Royal Society of Medicine (RSM) Winter meeting – London. "DNA aneuploidy and aberrant expression of RLP are tightly linked to clinical outcome in penile carcinoma". 2008

Annual Meeting of British Association of Urological Surgeons (BAUS) – Manchester. "Replication licensing factor Mcm2 predicts disease progression in penile cancer patients". BJU International, 97: 1–101. 2006.

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CHAPTER ONE

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CHAPTER ONE

GENERAL INTRODUCTION

1.1. Purpose and Objectives of the Thesis

Penile cancer is a rare urogenital malignancy with significant geographical variation and indeterminate aetiological factors (Daling et al. 2005; Barnholtz-Sloan et al. 2007; Curado et al. 2007). Current prognostic and predictive tools for the clinical management of men with penile cancer have limited discriminatory roles and radical surgery remains the cornerstone of treatment (Pizzocaro et al. 2010). The molecular mechanisms underpinning tumourigenesis and disease progression in penile cancer remain poorly understood (Muneer et al. 2009). Recent research in this area has focused on understanding the relationship between human papilloma virus (HPV) infection and tumour formation. However, this association is not absolute and other factors appear to be implicated. Despite improvements in surgical techniques and diagnostic methods, clinical outcomes in this difficult patient cohort have reached a plateau in recent times (Verhoeven et al. 2013; Djajadiningrat et al. 2013).

The efficacy of DNA replication licensing factors (RLFs) as novel biomarkers of diagnosis and prognosis has been described in various different cancer types (Blow and Hodgson 2002; Gonzalez et al. 2005; Laskey 2005; Blow and Gillespie 2008; Jackson, Laskey, and Coleman 2013). I have sought to investigate the role of these important effectors of cellular proliferation and differentiation in men with penile carcinoma (PeScc). Additionally, I examine how the dysregulation of these proteins

may contribute to the initiation and progression of this heterogeneous and complex malignancy. In Chapter 3, I investigate the histopathological patterns and clinicopathological features observed in men with penile cancer. Additionally, I correlate these data with tumour DNA content as a marker of genomic instability and assess the role of aneuploidy as a novel prognostic marker. In Chapter 4, using *in vitro* and *in vivo* model systems, I investigate the involvement of these cell cycle regulators in the transition from benign to malignant penile tissue. Furthermore, I investigate the multiparameter analysis of RLF expression to investigate the cell cycle kinetics of individual tumour specimens including penile squamous cell carcinoma subtypes. In Chapter 5, I show that the RLFs are intricately linked to the development of cell cycle dysregulation, genomic instability and the progression from indolent to aggressive phenotypes. Extending on these studies, in Chapter 6, I explore how the biological dysregulation of these G1/S regulatory proteins alongside aneuploidy impact on prognosis and survival in men with penile cancer.

1.2. Somatic Tissue Systems

1.2.1. Proliferative states define cellular sub-classes

Development and maintenance of human tissues in multicellular organisms requires precise spatial and temporal control of cellular proliferation. The major role of cell division in adult life is to maintain the number of differentiated, functional cells; thereby replacing cells that are lost through death or injury. Normal somatic tissue types can be broadly subdivided into three classes based on their growth control strategies and their ability to respond to mitogenic stimuli. These groups are defined as: self-renewing, stable and permanent tissues (Hall and Watt 1989; Potten and Loeffler 1990; Stoeber et al. 2001). The ability to arrest growth in quiescence,

differentiation and senescence reflects fundamental growth regulatory mechanisms that operate stringently in untransformed cells but are defective in tumour cells. In stable tissues such as thyroid and liver, all cells are differentiated but retain the ability to re-enter the cell division cycle in response to mitogenic cues ("dormant growth potential"). In contrast permanent tissues (e.g. neurones, cardiac myocytes) have no reserve capacity for growth in adult life (Leblond 1964). Tissues with the most rapid cellular turnover are referred to as self-renewing systems and include: skin, gut, testis and the haemopoietic system. These tissues share similar hierarchies of cellular development, from stem cells to terminally differentiated mature cells via transit amplifying cells (Figure 1.1) (Myster and Duronio 2000; Watt and Hogan 2000). Although the general scheme of cellular hierarchies is similar for self-renewing systems, differences are observed between those of somatic and germ cell type. In somatic self-renewing systems such as skin and gut, cell production occurs in the basal proliferative compartment through mitotic cell divisions with migration and differentiation of post-mitotic cells into the luminal compartment (Figure 1.2) (Potten and Loeffler 1990; Watt and Hogan 2000). In testis, a more complex cellular hierarchy is found. Germ cell development occurs in successive mitotic, meiotic and post-meiotic phases with germ cells moving from the basal compartment to the lumen of seminiferous tubules as cells engage their differentiation programme (spermatogenesis) (Potten 1986).

Neoplasia is regarded as disorder of cell growth and its origin is considered to result from somatic mutations and clonal selection, with successive collection of cells conferring growth advantages (Hanahan and Weinberg 2000). However, tumours can demonstrate variations in their patterns of growth and degree of differentiation. They

may arise from undifferentiated stem cells or from fully differentiated cells and can vary widely in their biological behaviour. It can therefore be postulated that abrogation of growth control

mechanisms in tumours derived from undifferentiated stem-like cells is likely to be different from deregulation in tumours derived from stable tissues. Squamous epithelium

appears to represent an appropriate "model system" to investigate tumour progression in self-renewing tissues. The specific attributes unique to penile squamous epithelium is introduced in more detail in section 1.5.1.

1.2.2. Epidermal organisation and hierarchy

Epithelial cells show specialised function and a capacity of self-renewal from their stem cell population and are organised into multi-layered structures such as the stratified epidermis, oesophagus, cervix and the lining of the mouth (Underwood J.C.E 1996). It is this organisation that is integral to their function as a protective interface between internal and external environments. The proliferative cells, including the stem cells that are ultimately responsible for renewing the epithelium, are anchored to an underlying basement membrane (Watt 1984). Cells that become committed to undergoing terminal differentiation, withdraw from the cell cycle, detach from the base membrane and move into the suprabasal layers. The epithelium of skin epidermis has a characteristic stratified appearance with keratinisation. As cells ascend from basal to apical regions, traversing the trans-amplification compartment, they undergo a progressive series of changes acquiring the characteristics that are required for their protective role (Figure 1.3).

Figure 1.1

Figure 1.2

Figure 1.3

Thus, it has been proposed that strict spatial correlation with regards to the basement membrane and also the solar polarity are key determinants in normal differentiation programs (Watt 2002). A continued, self-renewal of the cell population is essential as terminally differentiated cells eventually die and are shed from the outer surface. It has been proposed that epidermal turnover can be achieved over a 60 day period (Mackenzie 1997; Barthel and Aberdam 2005; Morasso and Tomic-Canic 2005).

1.2.3. Squamous epithelium: steps to carcinogenesis

1.2.3.1. Normal

Cellular proliferation and differentiation are under the influence and control of numerous local factors linked to mitogenic signalling pathways. These pathways are crucial in normal tissue repair processes and often malfunction in carcinogenesis.

Histologically, the differentiation process has defined three important layers within the epidermis (Underwood J.C.E 1996). Stratum basale is the lowest layer and contains the proliferative stem cell compartment. The cells located in the middle third of the epidermis, are recognisable squamous cells (keratinocytes) and are held together by desmosomes. Shrinkage of the cell and the presence of desmosomal bridges draw out small spines of cytoplasm from these cells giving them a typical prickle appearance from which they derive their name of stratum spinosum. During further migration towards the apical zone, these cells become simplified and metabolism becomes totally directed to producing the components of an eventually horny layer. The stratum corneum contains dead cells and leaves a highly structured keratin layer behind. Models have been developed describing the multistep progression from normal epithelium to malignancy through know intermediary steps

(Chisholm and Greene, Jr. 2011). These processes will be discussed further and the potential intermediary conditions highlighted.

1.2.3.2. Benign papilloma and squamous hyperplasia

A papilloma is defined as a benign tumour of non-glandular or non-secretory epithelium typified by squamous epithelium. The development of papillomas has been clearly described in other tumour types such colorectal cancer (Vogelstein and Kinzler 2004). Human papilloma virus (HPV) infection plays an important role in initiation of cutaneous malignancies and is detailed in section 1.5.4. Squamous cell hyperplasia is characterised by microscopic findings consisting of elongation, widening of the rete ridges and irregular thickening of the Malpighian layer of rete ridges (acanthosis), hyperkeratosis and chronic inflammation in the dermis. Parakeratosis may also be present. Inflammatory reactions within the dermis consist of lymphocytes and a small number of plasma cells. A number of premalignant penile dermatoses may reside under this umbrella term including: male genital lichen sclerosus et atrophicus (MGLS), penile cutaneous horn, leukoplakia, and pseudoepitheliomatous, keratotic and micaceous balanitis (PKMB) (Kayes, Shabbir, and Minhas 2012).

1.2.3.3. Dysplasia

Dysplasia can be defined as an observable expansion of immature cells in conjunction with a decrease in the number and location of mature cells. Dysplasia is often indicative of an early neoplastic process. The term dysplasia is typically used when the cellular abnormality is restricted to the originating tissue, as in the case of an early in-situ neoplasm. Dysplasia, in which cell maturation and differentiation are delayed,

can be contrasted with metaplasia, in which cells of one mature, differentiated type are replaced by cells of another mature, differentiated type (Underwood J.C.E 1996). Carcinoma in situ (Cis) represents a high grade dysplasia exhibiting all the features associated with malignancy but without evidence of invasion. Clinical examples which represent high risk premalignant, penile conditions include: Erythroplasia of Queyrat (EQ), Bowen's disease (BD) and Bowenoid Papulosis (BP) (Kayes, Shabbir, and Minhas 2012).

1.2.3.4. Squamous cell carcinoma

Squamous cell carcinomas are very common epithelial tumours with variable aetiological heritage. These tumours are characterised by focal or extensive loss of the basement membrane, an increased number of proliferating cells and a reduced number of cells that undergo terminal differentiation. In addition, the spatial organisation of cells is disrupted, leading to a loss of the normal multi-layered epidermal structure (Underwood J.C.E 1996). These disorganised keratinocytes demonstrate typical malignant cytology and also show foci of keratinisation within the tumour. The degree of keratinisation is utilised to define the level of differentiation demonstrated by these tumours, with well differentiated tumours expressing a greater proportion of keratin [Broder's grading system] (Broders AC. 1921). Integrin expression is often altered with focal, extensive integrin loss; especially with regards to basement membrane proteins and has been linked to metastatic capabilities (Janes and Watt 2006). Tumour progression often involves spread to locoregional lymph nodes and distant metastases can occur.

1.3. Cell Cycle Machinery

1.3.1. Cell cycle: Checkpoints, coordination and control

The cell cycle control system is the regulatory network that controls the order and timing of cell cycle events. The cell cycle consists of four coordinated processes: cell growth, DNA replication, distribution of the duplicated chromosomes to daughter cells and cell division. The process is essential to the survival and continuing viability of all cells. Regulatory factors ensure a tight control and coordination of the distinct phases of the cell cycle resulting in the accurate passage of the genome to daughter progenies (Underwood J.C.E 1996). Mitosis is the most dramatic stage of the cell cycle, corresponding to the separation of daughter chromosomes and resulting in cytokinesis. However, mitosis and cytokinesis last approximately one hour; thus 95% of the cell cycle is spent in interphase. At the molecular level, interphase is the time during which the cell cycle machinery undertakes the enormous task of duplicating each of the 3.2 billion base pairs that constitute its DNA (Alberts B, Johnson A, and Lewis J 2002).

The cell cycle is divided into four stages: G1, S, G2 and M phases. Cells initially begin cycling in the G1 phase, either directly from the M phase of a previous round of replication or from a state of quiescence (G0). G0 cells are metabolically active, although they cease to grow and have reduced rates of protein synthesis. Cycling cells have to pass through important regulatory checkpoints that ensure the successful completion of one stage of the cell cycle before being allowed to proceed to the next stage (Hartwell and Weinert 1989; Morgan 1997). The central components acting as biochemical switches driving phase to phase transition are the cyclin-dependent kinases (CDKs) (Nurse 2002). The duration of these cell cycle phases varies

considerably in different cell types. However, for a standard adult mammalian cell, the lengths of S-G2-M phases are relatively fixed with the greatest variability in periodicity observed during G1 phase. Due to physiological stresses and/or mitogenic cues, this stage of cell cycle can last a few minutes or as long as a few days.

A system of checkpoints and feedback controls are crucial in controlling the passage of cells through the cell cycle, maintaining high fidelity whilst stabilising replication forks and preventing cell cycle progression during replication stress or damage. The molecules which govern the G1/S and G2/M transitions are of crucial importance in dictating transit through the cell cycle and in preserving genomic integrity. Oscillations in the activity of critical 'cyclin-CDK' complexes at specific times during the cell cycle, govern the progression of cells through each stage (Hartwell and Weinert 1989; Morgan 1997). This process ensures that the cell cycle machinery is appropriately prepared for the next stage of the cell cycle. The precise timing of changes in CDK activity is overseen by multiple mechanisms. Fluctuation in the concentrations of corresponding regulatory cyclins is particularly important and is achieved through changes in cyclin gene expression and rates of cyclin degradation. Further modulation is achieved through the involvement of CDK inhibitory proteins. Cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 drive G1 progression through the restriction point (R), which commits the cell to complete the cycle (Planas-Silva and Weinberg 1997). S phase is initiated by cyclin A-CDK2, and cyclin B-CDK1 regulates progression through G2 and entry into mitosis (Nigg 2001). Progression through each cell cycle phase and transition from one phase to the next are monitored by sensor mechanisms, called checkpoints, which maintain the correct order of events. If the sensor mechanisms detect aberrant or incomplete cell cycle events (e.g. DNA

damage), checkpoint pathways carry the signal to effectors that can trigger cell cycle arrest until the problem is resolved.

The G1/S, S and M phase specific CDKs are inactive in G1, ensuring that cell cycle events are not triggered inappropriately before the cell commits to a new round of replication. In particular, a decision point in late G1 (restriction point ['R']) defines a temporal marker when the cell is committed to proceed through S phase and the rest of the cell cycle, even in the absence of further growth factor stimulation (Sclafani and Holzen 2007). Induction of molecules controlling this checkpoint is determined by increased gene expression through the E2F family of transcription factors. E2F function itself is regulated by the inhibitory retinoblastoma (Rb) pathway (Fang and Han 2006; Giacinti and Giordano 2006). The G1/S checkpoint defines a temporal marker for the cell to determine whether it should: divide, delay division or enter a resting stage (G0). For example, G1 arrest can be induced through the action of the Ink4 family [INK4A (p16), INK4B (p15), INK4C (p18) and INK4D (p19)] of CDKIs, which inhibit CDK4 and CDK6, or, alternatively, via the Cip/Kip family of inhibitors (p21, p27, p57), which suppress CDK2 activity (Malumbres and Barbacid 2009).This represents a fraction of the signalling pathways involved in cell cycle progression but highlight the complexity of the system identified to date.

These cellular control concepts are clearly illustrated in the elegant cell fusion experiments of Rao and Johnson in the 1970s, which provide insight into how cells maintain genomic stability with repeated rounds of replication (Rao and Johnson, 1970). When a G1 nucleus is fused to an S phase cell, the G1 nucleus begins to replicate prematurely. Therefore, a G1 nucleus is competent to replicate but lacks factors present in S phase that are necessary for DNA synthesis initiation. However,

delivering these factors to a G2 nucleus by fusing S and G2 phase cells fails to instigate replication in the G2 nucleus; indicating that the cell has some means of differentiating between replicated and unreplicated DNA. These observations have led to the concept of DNA replication licensing (Nishitani and Lygerou 2002; Blow and Hodgson 2002) and will be expanded on in the succeeding section.

Several other cell cycle checkpoints function to ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells. One of the most clearly defined of these checkpoints occurs in G2 and prevents the initiation of mitosis until DNA replication is completed (Foijer and Te 2006; Hook, Lin, and Dutta 2007). This G2 checkpoint senses unreplicated DNA, which generates a signal that leads to cell cycle arrest. Progression through the cell cycle is also arrested at the G2 checkpoint in response to DNA damage, such as that resulting from irradiation. This arrest allows time for the damage to be repaired, rather than being passed on to daughter cells. DNA damage not only arrests the cell cycle in G2, but also slows the progression of cells through S phase and arrests cell cycle progression at a checkpoint in G1. This G1 arrest may allow repair of the damage to take place before the cell enters S phase, where the damaged DNA would be replicated. In mammalian cells, arrest at the G1 checkpoint is mediated through p53 pathways, which is rapidly induced in response to damaged DNA (Sancar et al. 2004). Finally, a third important cell cycle checkpoint that maintains the integrity of the genome occurs toward the end of mitosis. This checkpoint monitors the alignment of chromosomes on the mitotic spindle, thus ensuring that a complete set of chromosomes is distributed accurately to the daughter cells (Musacchio and Salmon 2007). For example, the failure of one or more chromosomes to align properly on the spindle causes mitosis to arrest at metaphase prior to the segregation of the newly replicated chromosomes to daughter

nuclei. As a result of this checkpoint, the chromosomes do not separate until a complete complement of chromosomes has been organized for distribution to each daughter cell. Degradation of cyclin B up-regulates the anaphase-promoting complex (APC), leading to the breakdown of securin. Degradation via ubiquitination and subsequent proteolysis allows separase to separate sister chromatids. Other factors such as the mitotic engine kinases Plk 1, Aurora A and Aurora B are important factors in successful mitosis, controlling centrosome maturation and separation, chromosome orientation and segregation. (Hook, Lin, and Dutta 2007; Rieder 2011).

This brief overview of the mechanistic components both driving and regulating cell cycle progression in normal somatic cells permits insight into the potential steps in normal cell growth and division; which, when dysregulated, may result in genomic instability and tumuorigenesis. These areas will be reviewed in more detail in later sections and the current understanding of the molecular events driving carcinogenesis in PeScc explored.

1.3.2. Cell cycle: Cancer

Cancer is a disease in which cells no longer respond to the usual social signals that govern their behaviour in a tissue. This results in a new balance between the proliferation and survival of these cells, determined by alterations in mitogenic and apoptotic regulatory pathways. Tumour evolution is driven by the gradual accumulation of genetic mutations as a result of natural errors in the duplication, repair or segregation of DNA and chromosomes (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). However, the rate of spontaneous mutation in most human cells is insufficient to produce a large number of defects required for progression to malignant cancer. Therefore, tumourigenesis is thought to depend on

the acquisition of genetic instability: an increase in the rate at which genes and chromosomes are mutated, lost, amplified or re-arranged. This cellular phenotype will permit a significant growth and survival advantage over neighbouring somatic cells (Rajagopalan and Lengauer 2004; Storchova and Pellman 2004).

The cytogenic hallmarks of neoplastic change include: increased number of cells, abnormal cell size/shape, unusual cellular arrangement, increased nuclear content, abnormal chromosomes and mitotic figures. It is proposed that tumours escaping normal growth restraints and entering a hyperproliferative state with clonal expansion of neoplastic cellular populations, will do so through a multi-step process, whereby cells acquire numerous genetic mutations (Nowell 1976). Ultimately, the timing and combination of these defects in pathway regulation and gene expression can differ between tumour types. Colorectal cancer is a well-studied model for tumourigenesis that supports this hypothesis (Fearon and Vogelstein 1990; Jass 2006) and can be defined by a three step process: initiation, promotion and progression. Each stage reflects changes in either early or late molecular events which drive tumours to grow and spread. These events include: altered oncogene and tumour suppressor gene expression, epigenetic events, point mutations, checkpoint dysfunction, chromosomal abnormalities and telomere shortening.

Defects in mitogenic signalling lie at the heart of uncontrolled proliferation of tumour cells, through the activation of G1 and G1/S CDKs that drive entry into the cell cycle. Dominant oncogene mutations have been identified at nearly every step in these pathways in cancer cells. These steps include mutations in genes for mitogens establishing autocrine signalling pathways, cell surface receptors, downstream

effector molecules (i.e. Ras within the MAP kinase pathway) and transcriptional regulators (myc, E2F) (Massague 2004; Vogelstein and Kinzler 2004). Deregulation of the cell cycle engine underlies the uncontrolled cell proliferation that characterizes the malignant phenotype. Mitogens release the brakes of cell cycle progression by stimulating G1–S CDK activities, which trigger the phosphorylation of pRB proteins, leading to disruption of their interaction with the E2F family of transcription factors. In cancer cells, the pRB brakes are often defective, resulting in E2F dependent G1–S gene expression even in the absence of mitogens. This may arise as a result of activating tumourigenic mutations which have been identified in diverse tumours at all levels in the mitogenic signalling pathways from ligands and receptors (e.g. HER2/ErbB2/neu receptor mutations or HER2 gene amplification) to downstream signalling networks (e.g. Ras–Raf–MAPK or PI3K–Akt signalling pathways) and also for the cell cycle-regulated genes themselves (e.g. CYCLIND1 and CDK4 gene amplification) (Huang et al. 2002; Freier et al. 2003; Zhang, Yang, and Gray 2009). Aberrant signalling promotes activation of CDK–cyclin complexes, which phosphorylate Rb and attenuate its capacity to induce transcriptional repression. The notion that Rb phosphorylation is a convergence point for these oncogenic signalling pathways is consistent with the fact that inactivation of the RB gene by mutation or methylation is a common occurrence in cancer (Malumbres and Barbacid 2001). The inactivation of tumour suppressor genes that encode CDKIs (e.g. p15, p16 and p27) are also common events in diverse tumour types. This releases the brakes on cell cycle progression and further abrogation of checkpoint control mechanisms leads to the acquisition of genomic instability which drives tumour evolution (Malumbres and Barbacid 2001).

Genomic instability is defined by the loss of DNA repair mechanisms, chromosome instability (numerical and structural) and micro-satellite instability (MSI). It is imperative in normal, cycling cells undergoing DNA synthesis that tight control exists to ensure that re-replication of the genome does not occur. It is well understood that the CDK activity is central to this process (Storchova and Pellman 2004). Aneuploidy is a common finding in tumour cells and represents one of the hallmarks of cancer development. Whether aneuploidy is a 'cause or consequence' of cancer remains to be elucidated (Weaver and Cleveland 2007); however chromosomal instability can result through numerous aberrant mechanisms. Defective DNA damage response systems are central to the development of chromosomal instability, through large losses, amplifications or exchange of chromosomal segments. This is thought to occur as a result of abnormal mitosis or cytokinesis checkpoints leading to structural changes in parent DNA being passed to daughter cells (Kops, Weaver, and Cleveland 2005). Alternative mechanisms, resulting in imbalanced duplication of DNA, also include over-replication of single stranded DNA segments during S-phase (Hook, Lin, and Dutta 2007) and degeneration in telomere length and function in the presence of defective DNA damage repair systems (ATM/p53 and Akt/Bcl-2) (Feldser, Hackett, and Greider 2003). The key role of DNA replication licensing and the coordinated process of DNA replication in maintaining genomic stability will be discussed in the next section.

1.4. The DNA Replication Licensing Pathway

1.4.1. Background

The error-free duplication of the entire human genome, alongside its stable maintenance, is fundamental for normal cellular proliferation and survival. For this to occur, mammalian systems have developed a series of highly coordinated cellular pathways which behave in a strictly monitored fashion in order to regulate DNA replication. The initiation of DNA replication is a crucial decision point; lying at a convergent target of all mitogenic signalling transduction pathways that drive proliferation (Figure 1.4). In this respect, the DNA replication licensing pathway can be regarded as a "relay station" providing a molecular switch to couple growth regulatory pathways with the initiation of chromosomal replication (Stoeber et al. 2001; Blow and Hodgson 2002; Bell and Dutta 2002; Gonzalez et al. 2005; Barry and Bell 2006; Blow and Gillespie 2008). Furthermore, the dysregulation of this intricate replication apparatus has been shown to be linked to the development and progression of a diverse range of tumour states (Freeman et al. 1999; Meng et al. 2001; Stoeber et al. 2002a; Going et al. 2002; Gonzalez et al. 2003; Chatrath et al. 2003; Williams et al. 2004; Wharton et al. 2004; Shetty et al. 2005; Dudderidge et al. 2005; Obermann et al. 2005; Mehrotra et al. 2006; Scott et al. 2006; Kulkarni et al. 2007; Dudderidge et al. 2007; Mukherjee et al. 2007; Scarpini et al. 2008; Saeb-Parsy et al. 2012).

The current understanding of the molecular mechanisms involved in carcinogenesis includes a variety of mutations in key signalling pathways through mechanisms including: codon mismatch, gene silencing and point mutations leading to over-activity of oncogenic systems or the loss of regulatory tumour suppressor molecules (Knudson 2002). Tumours acquire a growth advantage over normal tissues through a

variety of mechanisms, including acquisition of aneuploidy and dysregulation of the mechanisms that control cellular proliferation. The DNA replication licensing pathway has emerged as a powerful downstream mechanism for controlling the proliferative state of cells and ensures that DNA is replicated once and only once per cell cycle; thus maintaining genomic fidelity (Blow and Hodgson 2002; Blow and Dutta 2005).

The initiation of DNA replication may provide an attractive therapeutic anti-cancer target by virtue of its position at the convergence of multiple signalling systems and since the mutational status of upstream signalling components should have no implication on the action of agents targeting this highly evolutionary conserved process. Studies demonstrating the exploitation of these proteins as prognostic, diagnostic and therapeutic targets will be reviewed in the subsequent sections.

Figure 1.4

1.4.2. Replication origins and DNA licensing initiation

In order to maintain eukaryotic genomic integrity, precise duplication of DNA must occur once, and only once, during each cell division cycle. This is achieved, during the G1-S transition, through tightly regulated initiation events at multiple replication origins scattered along each chromosome (Machida, Hamlin, and Dutta 2005). The basic mechanism of initiation occurs in several steps and results in the establishment of bidirectional replication forks at DNA origins:

(i) ***Origin recognition:*** label the origin with ORC.

(ii) ***Assembly of the pre-replicative initiation complex:*** load the DNA helicase to form the pre-RC.

(iii) ***DNA unwinding:*** activate the DNA helicase.

(iv) ***Elongative assembly:*** load the replisome including DNA polymerase and single stranded DNA binding protein.

A replication origin is a specific site on the chromosome where initiative proteins bind and open the DNA helix to allow DNA synthesis to begin (Figure 1.5). These sites on the DNA are commonly called the origin recognition complex (ORC) (Bell and Stillman 1992). Usually, replication begins at an origin and proceeds in a bidirectional manner to complete a single replicon.

The replication origins in mammalian cells are not distributed at regular intervals throughout the chromosomes but tend to be organised into clusters and are all activated simultaneously (Prasanth et al. 2004; Siddiqui and Stillman 2007). Approximately thirty thousand origins are postulated to exist in the mammalian genome; however, the number of licensed origins exceeds the number of origins activated in each cell cycle due to the assembly of pre-RCs at both active and silent origins. This acts to ensure that the entire genome is replicated even if some origins fail to initiate (Bell and Dutta 2002).

In higher eukaryotes the organisation of origins appears more complex and difficult to define compared to the models constructed in yeast. Human ORC is capable of binding functionally to any DNA sequence, which is consistent with the observation that replication can occur at multiple sites. Although the mechanism of ORC recruitment varies between eukaryotes, the subsequent steps involved in assembling the pre-RC after ORC binding are conserved among all eukaryotes. The replication origin forms the foundation on to which the pre-replicative complexes are assembled during late M and early G1 phases. Replication licensing factors: ORC, Cdc6, Cdt1 and Mcm2-7 assemble into pre-RCs, which render replication origins "licensed" for DNA synthesis (Blow and Hodgson 2002). This is a highly coordinated process with sequential loading of each factor onto the ORC subunit under the control of ATPase activity (Siddiqui and Stillman 2007).

Six paralogous proteins constitute the MCM complex, with Mcm2 to Mcm7 first identified in budding yeast; where defects in both initiation and elongation phases in

Figure 1.5

DNA replication were noted in mutated forms (Lei et al. 1997; Tye 1999; Forsburg 2004). Studies show that the six subunit MCM complex lacks helicase activity *in vitro*, suggesting that Mcm4/6/7 constitutes the core helicase while Mcm2/3/5 are regulatory subunits (Ishimi 1997; You, Komamura, and Ishimi 1999; Ishimi and Komamura-Kohno 2001). The model is supported by studies showing that the ATPase activity of the Mcm2-7 complex requires the coordinated action of all six subunits with Mcm4/6/7 involved in ATP hydrolysis and Mcm2/3/5 regulating its activity (Schwacha and Bell 2001). Consequently, the activation of MCM helicase activity, probably involves the re-arrangement of some of the units to form a ring-like molecule that encircles and unwinds the DNA (Davey et al. 2002). Each pre-RC constituent is essential for the initiation of DNA replication with reduced levels of any component leading to the loss of replicative capacity and an inability to progress into S-phase. Once MCM complexes are loaded onto chromatin, the other components of the pre-RC are dispensable for replication initiation. Therefore, the primary function of ORC, Cdc6 and Cdt1 in replication is to load the Mcm2-7 complex (Donovan et al. 1997; Hua and Newport 1998; Harvey and Newport 2003).

The transition from G1 to S phase involves the conversion of pre-RCs into replication forks. Initiation requires origin unwinding, stabilisation of single stranded DNA and loading of the replicative polymerases. These processes require the function of a second set of replication factors and the activity of at least two kinases – cyclin dependent kinases (CDKs) and Dbf4 dependent kinase (DDK); also termed Cdc7 kinase (Jiang et al. 1999; Grishina and Lattes 2005). These factors are temporally regulated throughout S phase and induce a conformational change in the pre-RC,

resulting in the recruitment of additional initiator proteins which collectively promote origin unwinding and recruitment of DNA polymerases.

The earliest initiation factor recruited to pre-RCs is Mcm10, which is involved in several critical functions. Mcm10 interacts with Mcm2-7, as well as ORC2 and Cdc7-Dbf4, and has been suggested to facilitate phosphorylation of Mcm7 by Cdc7-Dbf4 (Mendez and Stillman 2003; Takeda and Dutta 2005). The formation of an active helicase leads to the recruitment of additional factors, including Cdc45 and the four subunit GINS complex, which is also dependent on Cdc7 kinase activity (Gambus et al. 2006). Phosphorylation of the MCM complex by CDKs and DDK results in the recruitment of Cdc45 to the origin (Wohlschlegel et al. 2002a; Sawyer et al. 2004). In addition Mcm10 is required for the loading of Cdc45 onto the chromatin and like Mcm2-7 is essential for elongation of DNA synthesis. As well as activating the MCM complex the pre-initiation complex acts on the final part of DNA replication initiation by loading polymerase-alpha-primase and the other DNA polymerases delta and epsilon onto the DNA (Mimura and Takisawa 1998; Aparicio, Stout, and Bell 1999).

Once activated, the MCM helicase unwinds double-stranded DNA at origins to generate a single-stranded DNA template required to recruit the DNA synthesis machinery containing RPA, PCNA and DNA polymerase α-primase (Sclafani and Holzen 2007). Following entry into S phase, the licensing system is shut down to prevent re-initiation events at origins that have already been ‘fired’. The key event in suppressing relicensing of origins is the inactivation of the MCM loading factor Cdt1 through two mechanisms (Blow and Dutta 2005). First, Cdt1 undergoes cell cycle dependent proteolysis during S and G2 (Li et al. 2003). Second, residual Cdt1 is

inhibited by the binding of a small regulatory protein called geminin, which is expressed at high levels during the S, G2 and M phases (Tada et al. 2001; Wohlschlegel et al. 2002b).

1.4.3. Preventing re-replication errors through licence control

Cells have developed several mechanisms for avoiding re-replication of the genome through the control of DNA replication licensing and DNA synthesis. This is important for normal cell survival and may play a critical role in the generation and progression of abnormal neoplastic events. In mammalian cells these mechanisms impinge on the replication initiation factors Cdt1 and Cdc6 with the negative regulatory protein geminin. The interaction between geminin and Cdt1 in mammalian cells has been shown to prevent re-licensing of origins during S-G2-M (Blow and Dutta 2005; Li and Blow 2004a; Li and Blow 2005; Saxena and Dutta 2005). Both Cdt1 and ORC1 are subjected to phosphorylation dependent proteolysis in mammalian cells whilst phosphorylation decreases their affinity for chromatin and helicase activity. These processes are influenced by specific CDKs.

Geminin was initially identified in two independent functional screens in Xenopus (McGarry and Kirschner 1998; Maiorano, Rul, and Mechali 2004). It retains the ability to regulate DNA replication via its central Cdt1-binding coil domain and to modulate neural cell fate. The geminin molecule is a tetramer formed by two dimers with monomers interacting via coiled-coiled domains determined by both X-ray crystallography and electron microscopy studies (Okorokov et al. 2004). Geminin does not affect Cdt1 loading but rather inhibits recruitment of Mcm2-7 by Cdt1 on to chromatin by steric hindrance (Cook, Chasse, and Nevins 2004; Saxena et al. 2004).

Levels of geminin rise during S phase with Cdt1 binding during S, G2 and M phases to prevent the re-initiation of DNA replication. Geminin binds to Cdt1 and inhibits its activity until late M phase (Arias and Walter 2007) when geminin is degraded by the anaphase promoting complex (APC) via CDK-dependent ubiquitination and proteolysis (McGarry and Kirschner 1998; Li and Blow 2004b). A new round of DNA replication is initiated through inactivation of APC in late G1 and entry into the subsequent S phase (Benjamin et al. 2004).

The importance of geminin in regulating licensing and maintaining genomic stability has been demonstrated by knockdown experiments in Drosophila and mammalian cell lines, where geminin reduction results in re-replication and checkpoint activation that prevents entry into mitosis (Mihaylov et al. 2002; Melixetian and Helin 2004; Zhu, Chen, and Dutta 2004). Inappropriate replication licensing and initiation is linked to re-replication in cancer cells through the over-expression of Cdt1 and Cdc6 (Vaziri et al. 2003). The re-replicated regions are predominately enriched in segments of the chromosomes that replicate early in S phase and are accompanied by ATM/ATR and Chk2 dependent activation of p53 which is characteristic of DNA damage. The phenomenon is selectively observed in p53 negative cancer cells. This indicates that replication licensing is the main system that prevents the re-replication of DNA in a single cell cycle. Over-expression of Cdt1 or depletion of geminin results in the activation of various checkpoints, including the upstream ATM and ATR kinases and downstream Chk1 and Chk2 kinases leading to an arrest of the cell cycle and/or apoptosis (Melixetian and Helin 2004; Zhu, Chen, and Dutta 2004; Saxena and Dutta 2005).

1.4.4. *DNA replication licensing, proliferation and cancer*

As alluded to in previous sections (1.4.1 & 1.4.2); the DNA replication initiation machinery can be regarded as a final and critical step in growth control which is positioned at the convergence point of multiple, complex and branched upstream signalling pathways. This component of the cell cycle machinery therefore acts as a relay station, connecting growth signalling networks with the initiation of DNA synthesis, and is therefore a potentially attractive diagnostic and therapeutic target in the context of cancer (Gonzalez et al. 2005; Williams and Stoeber 2007; Williams and Stoeber 2012).

1.4.4.1. *Defining the proliferative state*

Investigation of the DNA replication initiation machinery in different organisms, tissues and cell types has revealed that cell cycle withdrawal and loss of proliferative capacity are linked to the shutdown of the licensing system (Blow and Hodgson 2002; Barkley et al. 2007; Kingsbury et al. 2005). It has been shown that during the proliferation differentiation switch, the MCM loading factors Cdc6 and Cdt1 are rapidly downregulated, as cells migrate from the transit amplifying compartment (TAC) to the functionally differentiated compartment of self-renewing tissues (Stoeber et al. 2001). This is coupled to a more gradual downregulation of the Mcm2-7 RLFs, as cells mature and adopt a fully differentiated phenotype. The conversion of replication origins into an unlicensed state also occurs during withdrawal from the cell division cycle into G0 quiescent and senescent “out-of-cycle” states as described in section 1.2.1. The repression of the DNA replication licensing machinery therefore appears to represent a ubiquitous and powerful downstream mechanism to restrain the proliferative capacity of cells in multicellular organisms and tissues following

withdrawal from the mitotic division cycle. It is interesting to note that many components of the DNA replication initiation machinery are under E2F transcriptional control (e.g. Cdc6, Cdt1, Mcm2-7, Mcm10 and Dbf4) and thus co-ordinates their downregulation during cell cycle withdrawal via the Rb-E2F pathway (Yan et al. 1998; Yoshida and Inoue 2004).

Proliferating cells have been shown to express high levels of the Mcm2-7 proteins throughout the cell division cycle [G1-S-G2-M]; with cyclical binding to origins occurring in late M/early G1 and displacement from chromatin during S phase (Stillman 1996). Consequently, Mcm2-7 proteins have emerged as novel biomarkers of proliferation. Unlicensed replication origins and absence of CDK activity, on the contrary, characterise the differentiated and G0 out-of-cycle states and therefore allow such cells to be clearly distinguished from cycling cells in complex and dynamic heterogeneous cell populations (Stoeber et al. 2001; Eward et al. 2004; Gonzalez et al. 2005; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013).

1.4.4.2. DNA replication licensing factors and cancer

RLFs represent novel biomarkers of growth and have emerged as powerful cancer biomarkers with clinical utility is a wide range of settings including cancer detection and screening, monitoring of therapeutic response, tumour surveillance and prognostication (Williams and Stoeber 2007). Notably it has been shown that dysregulation of the DNA replication licensing machinery is an early event in multistep tumourigenesis occurring at the precursor premalignant stage of cancer development (Freeman et al. 1999; Williams et al. 1998; Kodani et al. 2001; Going et al. 2002; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013).

Identification of MCM proteins in pathological specimens using immunodetection methods has been shown to be an accurate and simple method for determining the growth fraction in dynamic tumour cell populations (Williams et al. 1998; Freeman et al. 1999; Going et al. 2002; Alison, Hunt, and Forbes 2002). Moreover, Mcm2–7 expression levels are powerful prognostic indicators in diverse tumour types, including cancers of the lung, breast, kidney, bladder, prostate and ovary (Meng et al. 2001; Gonzalez et al. 2003; Shetty et al. 2005; Dudderidge et al. 2005; Korkolopoulou et al. 2005; Kulkarni et al. 2007). This finding is consistent with large-scale meta-analysis of cancer microarray data, which identified up-regulation of the MCM2–6 genes as a component of poor prognostic signatures (Rhodes et al. 2004). In most tumour types, the up-regulation of MCM and other licensing proteins is likely to reflect oncogene-driven engagement of the cell division cycle. However, deregulation of the licensing system may also be a primary driver of oncogenesis, at least in some tumour types. For example, over-expression of Cdc6 or Cdt1 have been shown to be oncogenic, and deregulated Mcm7 expression has been linked to tumour formation, progression and malignant transformation in animal models (Arentson et al. 2002; Seo et al. 2005; Liontos et al. 2007; Shima et al. 2007; Pruitt, Bailey, and Freeland 2007). Oncogenic mutations in genes upstream of the licensing machinery (e.g. RAS, CYCLINE and CYCLIND1) can also impact on tumourigenesis by causing deregulation of the licensing machinery. This may either result in relicensing events or allow cells to enter S phase with insufficient licensed origins, both of which can lead to genomic instability (Blow and Gillespie 2008).

1.4.4.3. Tumour cell cycle phase analysis

Tumour cell cycle kinetics has been shown to impact on not only prognostic assessment but also response to cell cycle phase specific chemotherapeutic agents. Indeed prognostic algorithms for many tumour types include parameters which are linked to the proliferative potential of the tumour. Such parameters include the assessment of the mitotic index and/or Ki67 labelling index. Prognostic algorithms used in common practice which incorporate a measurement of proliferative potential include: the Nottingham Prognostic Index (NPI) for breast cancer (Elston and Ellis 1991) and the Federal Nationale Des Centresde Lute le Cancer Grading System for Soft Tissue Sarcoma (Coindre 2006). However, these biomarkers represent crude measurements of proliferation and are compromised. For example fixation delay of tissue can influence the assessment of mitotic index. Although Ki67 has emerged as a promising prognostic marker, its routine introduction into clinical practice has been vitiated by conflicting data from meta-analysis studies (Yerushalmi et al. 2010). Moreover, harmonization in methods used to quantify Ki67 levels between laboratories has proven problematic and reported cut-points vary widely. Additionally, although Ki67 has now been utilised for over 30 years as a proliferation marker, its function remains poorly defined (Scholzen and Gerdes 2000). In contrast the function of the RLFs has been extensively studied in a wide variety of species and, therefore, offers a new class of biomarkers for the assessment of proliferation in human biopsy material.

The analysis of core constituents of the cell cycle machinery now provides an alternative method to assess the proliferative state of dynamic tumour cell populations. As discussed above expression of the Mcm2-7 proteins allows tumour

cells engaged in the mitotic cycle to be clearly distinguished from cells residing in terminally differentiated, quiescent (G0) and senescent "out-of-cycle" states. MCMs are expressed at high levels during the G1-S-G2-M phases of the cell division cycle contrasting with their tight downregulation in terminally differentiated/G0 cells. The expression of geminin in contrast is restricted to the S-G2-M phases of the cell division cycle and importantly is indicative of cell cycle progression (Wharton et al. 2004). As discussed above Ki67, a 345 kDa protein is expressed in actively proliferating cells in all proliferating cells in all cell-cycle phases (G1, S, G2, and M). It has been shown in multiple tumour types and tissues that combinatorial analysis of these cell cycle biomarkers (i.e. Mcm2-7, geminin and Ki67) and more recently with the M phase marker phosphohistone H3(H3S10ph) can provide more detailed information regarding the cell cycle kinetics of complex dynamic cell populations *in vivo* (Kulkarni et al. 2007; Loddo et al. 2009).

Multiparameter analysis using this set of biomarkers has revealed new insights to proliferation control in stable, self-renewing and permanent tissues and the tumours arising from these tissues. For example immunostaining of colonic epithelium and other self-renewing tissues such as cervical and oesophageal epithelium has revealed an inverse relationship between DNA replication licensing and differentiation in these tissues (Stoeber et al. 2001). Mcm2-7 levels are expressed at high levels in the transit amplifying compartments of these tissues indicating a high proportion of these cells are engaged in the cell division cycle. In contrast surface terminally differentiated cells are MCM negative in keeping with *in vitro* studies that the removal of the replication licence is a common pathway by which proliferation is restrained (Musahl et al. 1998; Kingsbury et al. 2005; Barkley et al. 2007; Williams and Stoeber 2012).

The MCM negative status is indicative that these cells have withdrawn from the proliferative cycle. Notably the high proportion of the MCM positive cells in the transit amplifying compartments of these tissues are associated with high levels of geminin, indicating that the cells are actively progressing through cycle (S-G2-M) fraction. Intriguingly resting mammary epithelium and ovary have been shown to demonstrate high levels of expression of the Mcm2-7 proteins but lacks expression of either geminin or Ki67 indicating these cells reside in a G1 state (Stoeber et al. 2001; Eward et al. 2004). In contrast pregnant, proliferating breast epithelium expresses high levels of all three biomarkers indicative of active cell proliferation. In contrast, secretory- differentiated, lactating breast tissues show a marked downregulation of Mcm2-7, geminin and Ki67 indicating withdrawal from the cell division cycle. Interestingly, the licensed G1 arrested/delayed state observed in normal resting mammary epithelium has been observed in precursor dysplastic lesions of self-renewing epithelia including: cervix, bladder and oesophagus (Williams et al. 1998; Going et al. 2002; Stoeber et al. 2002b; Williams and Stoeber 2012). The arrested differentiation in these precursor lesions is associated with high Mcm2-7 expression but expression levels of Ki67 and geminin are very low indicating the majority of these cells reside in a G1 arrested/delayed state. It has also been shown that the majority of tumour cells in more advanced invasive cancers arising from these tissues also reside in this licensed G1 arrested state. The added sensitivity of Mcm2-7 biomarkers over Ki67 has been exploited in the development of Mcm2-7 cancer detection tests for a number of organ systems including: cervix, bladder and prostate (Williams et al. 1998; Meng et al. 2001; Stoeber et al. 2002a; Dudderidge et al. 2010; Kelly et al. 2012). The clinical utility of these Mcm2-7 biomarkers has now reached the advanced stage of commercialisation including validation studies for FDA

approval. For example, Tripath/Beckton Dickinson have developed the ProEx C test for improved detection of abnormal cells in the cervical Pap smear test [http:www.bd.com/tripath/products/proexc]. This exploits the use of MCM biomarkers for the improved detection of precursor malignant cells using an immunocytology approach. Similarly, Cytosystems Ltd [http:www.cytosystems.com] and Urosens Ltd [http:www.urosens.com] have developed Mcm2-7 assays for the detection of bladder and prostate cancer. It is interesting to speculate that the persistence of Mcm2-7 in non-proliferating breast may be an evolutionary relic from times when women spent most of their fertile years either pregnant or lactating and consequently, it was therefore unnecessary for these cells to be able to withdraw from the G1 state. An important question raised by this finding is whether licensed but slowly proliferating cells such as these have a higher risk of undergoing malignant transformation. Since the lack of origin licensing may be an important mechanism restraining the proliferation of G0 cells, it is possible that failure to down-regulate the licensing system (as in these breast cells) may make transition to uncontrolled proliferation significantly easier to achieve. Taken together these extensive clinicopathological studies have revealed that multiparameter analysis with these novel cell cycle biomarkers can be used to identify three discrete cell cycle states in complex dynamic tissues: (i) an "out-of-cycle", unlicensed state characterized by an absence of expression of Mcm2-7, geminin and Ki67, (ii) an actively cycling state characterized by high expression levels of the Mcm2-7 proteins, geminin and Ki67 and (iii) G1 arrested/delayed state characterized by high Mcm2-7 expression levels but low geminin and Ki67 levels. This latter cell population has been referred to as "licensed cells with proliferative potential" (reviewed in Figure 1.6).

Figure 1.6

Additional cell cycle parameters have also been identified using combinatorial analysis of these three cell cycle biomarkers in relation to tumour cell cycle kinetics (Wharton et al. 2004; Dudderidge et al. 2005; Kulkarni et al. 2007; Loddo et al. 2009). As discussed above, the Ki67 labelling index identifies all phases of the proliferative cell cycle (G1, S, G2, M) and the geminin labelling index identifies the fraction of cells in S–G2–M. The geminin/Ki67 ratio is therefore an indicator of the relative length of the G1 phase. Proliferating cells with a short G1 phase will approximate to a geminin/Ki67 ratio of ~1; whereas cells with a prolonged G1 phase will approximate to a geminin/Ki67 ratio ~0. Studies have shown that tumours with a more aggressive phenotype are associated with high geminin/Ki67 ratios. Alternatively, the Mcm2-7/Ki67 ratio can be used to define the proportion of cells that are licensed to proliferate, representing a ratio of licensed to actively proliferating cells. Thus, the higher the Mcm2/Ki67 ratio then the greater the proportion of cells that reside in a licensed, non-cycling state. This has been shown to be an important parameter of biological and prognostic significance in a range of different tumour types as described above. Moreover the difference in the labelling index between Ki67 and geminin [Ki67-geminin] can be used to estimate the percentage of cells that are transiting G1 (i.e. G1 growth fraction). Overall, this information is of potential value in determining the cell cycle kinetics of dynamic tumour cell populations in normal and malignant penile epithelium. I investigate RLF expression and associated multiparameter tumour cell cycle phases kinetic analysis in benign and malignant penile tissues in section four. Furthermore, in later chapters, I link these data to clinical and prognostic information to determine their utility as novel biomarkers in men with penile cancer.

1.5. Penile Squamous Cell Carcinoma (PeScc)

1.5.1. Penile anatomy: relevance to staging and surgery in PeScc

The penis consists of three separate cylindrical structures and originates from the genital tubercle at the cranial end of the cloacal membrane in utero. Normal development is under the direct control of androgenic hormonal stimulation. The penis is composed of three parts: the shaft/body, anterior (glans, corona, foreskin) and posterior (perineal attachments). The corpora cavernosa join beneath the pubis to form the major portion of the body of the penis. These two cylinders of the penis are separated by a septum which allows the vascular spaces to freely communicate. They are enclosed by the tough tunica albuginea, which is predominantly collagenous and meets distally to separate the heads of the copora cavernosa from the corpus spongiosum of the glans. It completely encases the copora cavernosa and appears to constitute an important biological barrier to the dissemination of PeScc (Velazquez et al. 2004; Leijte et al. 2008; Heyns, Mendoza-Valdes, and Pompeo 2010).

The corpus spongiosum tapers and runs on the underside of the corpora cavernosa and then expands to cap them as the glans penis. The corona separates the base of the glans from the shaft of the penis. The spongiosum is traversed throughout its length by the anterior urethra, which begins at the perineal membrane. Proximally, it is lined by stratified and pseudostratified columnar epithelium, distally by stratified squamous epithelium. The mucus-secreting glands may be seen as small outpouchings of the mucosa.

Buck's fascia surrounds both cavernosal bodies dorsally and splits to surround the spongiosum ventrally. This important layer can clearly be defined on MRI and provides crucial information for surgeons pre-operatively, when planning

conservative surgical techniques (Kayes et al. 2007). In the perineum, Buck's fascia fuses with the tunica albuginea deep to the muscles of the erectile bodies. Distally, it fuses with the base of the glans at the corona.

The skin of the penile shaft is highly elastic and without appendages (hair or glandular elements), except for the smegma-producing glands at the base of the corona. Distally, it folds over the glans as the foreskin (double-membrane) and attaches firmly below the corona. Its blood supply is independent of the erectile bodies and is derived from the external pudendal branches of the femoral vessels. These vessels enter the base of the penis to run longitudinally in the dartos fascia as a richly anastomotic network. The skin of the glans is immobile as a result of its direct attachment to the underlying, thin tunica albuginea (Heitz, Pottek, and Schreiter 1998; Velazquez et al. 2003).

A rich network of lymphatics of the glans and the copora cavernosa courses along the dorsal vein to the symphysis pubis and drains into superficial and deep inguinal lymph nodes which are anatomically separated by the deep fascia of the thigh (fascia lata). The superficial group of nodes (12 to 15 nodes) are situated in the deep membranous layer of the superficial fascia of the thigh (Camper's fascia). The sentinel lymph node group (1 to 3 nodes) can be identified by their anatomical location at the anterior or medial aspect of the superficial epigastric vein (Cabanas 1977; Cabanas 2000). The node of Cloquet is the most cephalad of the deep group and is situated within the femoral canal (Shen et al. 2000). The external iliac lymph nodes receive drainage from the deep inguinal, obturator, and hypogastric groups. In turn, drainage progresses to the common iliac and para-aortic nodes. The earliest route of dissemination in penile carcinoma is to loco-regional lymphatics. Penile carcinomas

have a predictable pattern of local, regional and systemic spread (Cabanas 1992; Horenblas et al. 1993). Locally invasive tumours initially penetrate the lamina propria, supeficial dartos or superficial corpus spongiosum layers (Figure 1.8). Later invasion involves deep corpus spongiosum, tunica albuginea, corpora cavernosa and preputial skin (Figure 1.9). Urethral invasion is not unusual in early or late stages depending on the site of origin of the initial tumour. Advanced tumour with local tissue destruction often represent higher staged and aggressive disease states (Figure 1.10), Intrapenile satellitosis (nodules of carcinoma separated from the main tumour mass) is a late phenomenon related to aggressive tumour behaviour (Velazquez et al. 2005).

Figure 1.7

Figure 1.8

Figure 1.9

Subsequently, loco-regional involvement occurs in a step-wise fashion, involving initially the superficial inguinal nodes before spread occurs to the deeper inguinal layer and finally onto pelvic and distant sites. It is extremely rare to observe disease 'skipping' nodal stations. The presence of lymphatic metastases strongly impacts on prognosis.

Patients with locally controlled disease have five year overall survival rates of greater than 90%; decreasing dramatically to approximately 30-50% if lymph node positive. There is a striking deterioration in survival outcomes with the increasing number of nodes involved and also the level of nodal involvement. Currently, 5 year survival is 0-38% from pelvic metastases in published series (Ravi 1993; Ornellas et al. 2008).

It has been shown that early lymph node clearance compared to delayed/therapeutic dissection, conveys a clear survival advantage (Kroon et al. 2005a; McDougal 2005). However, patient selection is notoriously difficult, as current staging techniques lack the required accuracy (section 1.5.3). Additionally, radical regional lymph node dissection carries high rates of mortality (3%) and morbidity (50-90%) (Horenblas et al. 1993; Pizzocaro, Piva, and Nicolai 1996; Sanchez-Ortiz and Pettaway 2004). Complications encountered may include: wound infections, wound dehiscence, flap necrosis, disabling lymphoedema and seroma formation.

This clinical paradigm highlights the current limitations that clinicians and scientists face when treating men with PeScc. Novel management algorithms should aim to stratify patients appropriately to: (i) surveillance programmes, (ii) aggressive surgical protocols and (iii) adjuvant chemotherapeutic treatments. It is important that novel biomarkers accurately reflect the biological status of the disease and predict disease

progression for high risk patients. Overall, future discoveries should ultimately minimise unnecessary co-morbidity and impairment to the patient's quality of life whilst improving overall survival.

1.5.2. Epidemiology and aetiology

Penile cancer is a rare neoplasm with approximately 400-500 new cases and 80-100 deaths annually in the United Kingdom. In Western countries, primary malignant penile cancer is uncommon, with an incidence of less than 1.00 per 100,000 males in Europe and the United States (Barnholtz-Sloan et al. 2007; Cancer Incidence in Five Continents. Vol. IX. IARC Scientific Publication, No. 1602009). However, there are significant geographical variations, with some centres in Europe reporting an incidence greater than 1.00 per 100,000 men (Parkin et al. 2005). In contrast, in the non-Western world, the incidence of penile cancer is much higher and can represent 10-20% of malignant diseases in men ranging from an age-adjusted incidence of 0.7-3 per 100,000 people in India to 8.3 per 100,000 men in Brazil, and even higher in Uganda, where it is the most commonly diagnosed male cancer (Owor 1984; Wabinga et al. 2000). Important risk factors include social and cultural habits alongside hygienic and religious practices.

Squamous cell carcinoma (PeScc) is the predominant tumour variant accounting for 95% of cases (Burgers, Badalament, and Drago 1992). Other malignant tumour types described in the literature include: adenocarcinoma, lymphoma, melanoma and various mesenchymal tumours such as Kaposi sarcoma and leiomyosarcoma. Initial presentation usually occurs in the sixth decade of life, often with areas of ulceration on the distal penis. Associated infection can present with offensive discharge and

cases of auto-amputation have also been reported (Rajaian, Gopalakrishnan, and Kekre 2010). Despite the fact that causation has yet to be fully delineated, a number of risk factors have been identified. It is well known that neonatal circumcision appears protective (Schoen 1996; Daling et al. 2005), as denoted by the extremely low incidence of penile cancer in Jewish populations. The protective mechanisms of circumcision are lost if surgery is delayed, thus establishing the hypothesis that factors within the inner preputial environment promote carcinogenesis. This hypothesis is supported by the increased frequency of preputial stenosis (phimosis) witnessed in uncircumcised patients presenting with penile cancer.

Other important risk factors include: inflammatory conditions (balanitis xerotica obliterans), human papilloma virus (HPV) infection and smoking (Moore et al. 2001; Pietrzak et al. 2006; Kayes, Shabbir, and Minhas 2012). The combination of these factors suggests that this malignant process involves multiple molecular pathways, which may be altered by the presence of chronic inflammation, oncogenic viral products and carcinogenic cofactors. Two core studies conducted by the IARC have reviewed the prevalence of HPV infection and cancer developments between stable couples. Their findings support a decreased prevalence of HPV infection in circumcised men (Castellsague et al. 2002) and that the incidence of cervical cancer was increased in uncircumcised men with a history of multiple sexual partners (Franceschi et al. 2002). These studies failed to elucidate on the long infective latency times that are observed in cancer development and evaluating time of HPV transmission. The various pathways involved in tumourigenesis and progression in PeScc are discussed in detail throughout this section and a hypothetical model for

carcinogenesis in penile cancer through HPV dependent and independent mechanisms is proposed (Figures 1.10 and 1.11).

1.5.3. Diagnosis, staging and prognostic factors

Patients should be examined to evaluate clinically advanced tumours; recording the location of the primary tumour, tumour size, involvement of penile/adjacent structures and the presence of inguinal lymphadenopathy. Confirmation of PeScc on biopsy material is mandatory and patients are staged through the routine use of pre-operative CT scanning. Penile MRI can help to delineate local staging of the primary tumour (see section 1.5.1) and assist in planning surgical strategies. These measures are important in contemporary surgical practice as radical penile amputation carries significant psychological and physical morbidity. In appropriate cases, where tumours are located distally and superficially on the penis (Ta, T1 & T2 without cavernosal involvement), the implementation of conservative surgical techniques have been successfully employed thus preserving phallus length and function in these patients (Minhas et al. 2005).

As discussed in the previous section, disease progression is in a loco-regional fashion, with sequential involvement of inguinal and pelvic lymph nodes; before distant metastases develop. Common sites for distant spread include: lung, brain and bone. Tumour recurrence can develop locally, within the lymphatic basins, subcutaneously with discrete nodules or at the distant sites already alluded to. Death is usually from inanition and sepsis; although catastrophic bleeding as a result of erosion of the femoral vessels caused by fungating, inguinal metastases can occur.

Figure 1.10

Figure 1.11

Recent studies have suggested that in the presence of palpable inguinal lymphadenopathy, the probability of inguinal metastases is approximately 80% (Hegarty et al. 2006). This supports recent changes in clinical practice and guidance (Pizzocaro et al. 2010), such that patients should be counselled for immediate modified or radical inguinal lymphadenectomy rather than delaying surgical intervention for a period of antibiotic therapy to exclude infective or inflammatory lymphadenopathy. Improvement in the five year survival rates following early surgical clearance of positive nodal disease provides strong evidence to support the implementation of radical surgery in this clinical setting (Kroon et al. 2005a; McDougal 1995).

However, treatment protocols for patients with clinically negative examination findings remains controversial; as up to 20% of these patients will harbour micro-metastatic disease that will be missed should the patients be entered into a surveillance program (Kroon, Horenblas, and Nieweg 2005). In order to improve patient selection to either surveillance, surgical and/or chemotherapeutic regimes important predictive information can be gained from primary tumour histology (Cubilla 2009). The single most important prognostic factor for overall outcome is nodal status; with 5 year survival figures of 90-100%, 80-90%, 40-50% and 0-30% for N0 (negative nodes), N1 (single superficial, inguinal lymph node), N2 (multiple or bilateral superficial inguinal lymph nodes) and N3 (deep inguinal or pelvic lymph node[s], unilateral or bilateral) respectively (Ravi 1993; Horenblas et al. 1993). Current histopathological features with prognostic significance for predicting loco-regional spread and overall survival include: grade, stage, subtype, depth of invasion, lymphovascular invasion and tumour size (Ficarra et al. 2006; Kattan et al. 2006;

Lopes et al. 1996; Ornellas et al. 2008). This information has helped shape European guidelines for the management of suspected inguinal metastases by identifying patients at high risk of developing regional spread (Pizzocaro et al. 2010). Patients can be stratified into risk groups; with higher risk patients selected for radical surgical treatment based on tumour grade and stage. Lower risk groups can be entered into surveillance programs with clinical and radiological examination at regular intervals.

Alternative approaches for staging include the use of sentinel node biopsy techniques with improving expertise in larger centres with greater experience (Hadway et al. 2007; Kroon et al. 2005b). The technique involves injection of nanocolliod radionuclide solution 24 hours prior to surgery and "hot spots" noted using a gamma camera and probe. The use of toludine blue dye injected peri-tumourally aid in the guidance and mapping out of lymphatics at the time of surgery to help isolate the sentinel node (i.e. first draining node in the primary lymphatic echelon). Removal of the lymph node for histopathological analysis is performed and if metastasis discovered, then formal radical lymphadenectomy is performed. Current sensitivities for this technique are variable with UK experience around 80% (Hadway et al. 2007), however Dutch 10 year experience is higher at 90% (Kroon et al. 2005b).

1.5.4. Human papilloma virus: Cause or cofactor?

Human Papilloma Virus (HPV) is a family of small double-stranded DNA viruses (8000 bp) with distinct pathogenetic types. Sexual transmission is the most common route for viral propagation, although both oral and vertical transmission has been described. High risk HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68 and 69 whilst low risk types are 6, 11, 26, 30, 34, 40, 42, 43, 44, 53, 55, 57, 61, 62, 64, 67, 70, 71, 73, 74, 79, 81-84 (zur 1990; zur 2000). Viral genotypes have conserved genetic, transcriptional and functional organisation, with early (E) regions encoding proteins for replication, regulation, and modification of the host cytoplasm and nucleus and a late (L) region encoding capsid proteins.

HPV has a strong correlation with the carcinogenic processes involved in anogenital tumour formation (zur 1990). Studies assessing the prevalence of high risk types and the association of oncogenic proteins E6 and E7 in the pathways controlling cellular differentiation, proliferation and apoptosis have provided evidence that HPV infection is a key player in the generation of in-situ and invasive cancers of epithelial tumours (Kamath et al. 2000; Palmer et al. 1989; Walboomers et al. 1999). Squamous cell carcinoma of the uterine cervix has provided the most convincing model for understanding the molecular alterations seen with HPV infection and HPV DNA integration into the human genome. Recent advances have led to the manufacture of the first HPV specific, bivalent and quadrivalent vaccines, which in clinical trials has demonstrated efficacy in preventing infection (Harper et al. 2006; Villa et al. 2006). The benefits of this vaccine for other anogenital and aerodigestive HPV-related malignancies are yet to be evaluated.

Fundamental understanding of HPV mechanisms during infection and viral replication has been extensively reviewed (Hebner and Laimins 2006). The overall prevalence of HPV DNA in penile carcinoma ranges from 20-80%, dependent on methodological approaches and geographical variance (Wiener et al. 1992; Villa and Lopes 1986; Lam et al. 1995; Picconi et al. 2000; Rubin et al. 2001; Daling et al. 2005; Senba et al. 2006). Implementation of advanced PCR techniques has generated a reference figure of approximately 40-45%, which is similar to the detection rates seen in vulval cancer (Della et al. 1992). Notably, the prevalence of HPV DNA detection in cervical neoplasia is 100% for in-situ disease and 99.7% for invasive tumours (Walboomers et al. 1999).

Distinct pathological variants have been identified which are associated with indolent behaviour (verrucous, warty, Buschke-Lowenstein condyloma) and others with more aggressive phenotype (Squamous cell carcinoma of usual type [NOS], basaloid, papillary) (Cubilla et al. 1993; Cubilla 1995). It has been demonstrated that there is a correlation between tumour subtype and HPV infection. Basaloid and warty variants are regularly HPV-associated (80-100%), demonstrating koilocytic changes on standard microscopy; whereas only a third of keratinising and verrucous tumours have such a relationship (Gross and Pfister 2004). It appears that carcinoma in-situ is strongly related to high risk HPV infection (90%) (Gross and Pfister 2004). HPV 16 and 18 are the most common types isolated in specimens of carcinoma in-situ and invasive PeScc (60-75%) whilst HPV 6 and 11 are most frequently seen in lower risk condylomas and Buschke-Lowenstein tumours. Verrucous tumours appear negative for HPV involvement, with only individual isolated cases showing positivity for the virus (Dianzani et al. 1998). This has led to the hypothesis that only a subset of

tumours is HPV-dependent with the remainder subject to other genetic and molecular alterations.

Two viral genes, E6 and E7, are continuously expressed in high risk types of the HPV transformed cells. E6-E7 gene products cooperate in disturbing cellular differentiation, proliferation and apoptosis through their involvement with the retinoblastoma Rb/E2F and p53 tumour suppressor pathways. Certainly, their expression is required to induce and maintain the neoplastic phenotype of cervical cancer cells and similar mechanisms seem to exist in penile cancer. Such alterations in these pathways which have been described in HPV positive and negative patients with penile cancer are reviewed subsequently.

The prognostic influence of HPV infection in patients with PeScc is controversial owing to contradictory evidence. Traditionally, it has been proposed that high risk HPV types, particularly HPV-16 and 18 are associated with aggressive variants and therefore give rise to poorer survival. Chan et al reviewed HPV 16/18 infection in a cohort of men with PeScc from Hong Kong (Chan et al. 1994). The data suggested that verrucous and well differentiated tumours were not associated with high risk HPV types. However, the specific impact of high risk HPV types in higher grade tumours was not commented upon. Gregoire and colleagues have supported the hypothesis that HPV 16/18 infections are highly associated with specific tumour types (basaloid/warty) which have been identified as aggressive variants (Gregoire et al. 1995). They also demonstrated that HPV-infected tumours are linked to higher grade and aggressive growth patterns. These results are confirmed by the presence of HPV 16 and 18 types in a number of cases with positive inguinal metastases (Wiener et al.

1992; Scinicariello et al. 1992; Leis et al. 1998). Weiner et al have demonstrated in a retrospective study the potential link of HPV 16 infection to disease progression (Wiener et al. 1992). However, they were unable to demonstrate a survival difference in patients with HPV compared to HPV negative cases.

More recent evidence for HPV 18 involvement in the metastatic process in penile cancer cases from Thailand has been published. This aggressive phenotype was found in 4 out of five cases with metastatic disease (Senba et al. 2006). High HPV 18 infection rates are witnessed in Brazil and may explain the geographic differences seen in South America. Bezerra et al reviewed similar parameters and outcomes following primary penile amputation and bilateral lymphadenectomy in 82 patients and revealed no correlation to lymph node metastases or survival outcome at 10 years (Bezerra et al. 2001). Their data suggest that HPV infection does not influence prognosis in invasive PeScc. Interestingly, over half (51.2%) of the patients were found to have inguinal metastases, but the series only contained one poorly differentiated tumour.

The largest series examining the relationship of HPV infection with prognosis (171 patients) found no association to lymph node metastases (Lont et al. 2006). On the contrary, there seemed to be a positive impact on outcome with high risk HPV subtype infection conferring a survival advantage with 5-year survival at 78% versus 93% in HPV-negative and HPV-positive patients, respectively. These data propose that whilst HPV infection can be linked to a distinct subset of carcinogenic events in men with penile cancer, the involvement of the viral genome may be protective against the development of aggressive variants. Such a correlation with lymph node

status and prognosis suggest alternate mechanisms for tumour invasion and metastases. Nonetheless, the interaction between HPV and penile cancer development is far from straightforward and requires further investigation in preclinical models.

1.5.5. Molecular biology of penile cancer

1.5.5.1. Karyotype profiling, ploidy status and telomerase activity

Genetic imbalances and karyotypic findings have been reported in penile cancer (Ornellas et al. 1998; Ornellas et al. 1999; Alves et al. 2001b), through cytogenetic analysis and genomic hybridization techniques. The rarity of penile cancer alongside technical difficulties inherent in this tumour due to the presence of inflammation, necrosis and poor growth characteristics in culture have led to a relative paucity of data linking chromosomal abnormalities to biological behaviour and outcome. Alves et al noted that DNA sequence copy number alterations (CNAs) were detected with distinct similarity to oral Scc and oesophageal Scc. In 23 cases analysed, common copy number gains included 8q24, 16p11-12, 20q11-13, 22q, 19q13 and 5p15. Deletions were found in 13q21-22, 4q21-32 and along the X chromosome. No association to grade or stage was detected, but there was a tentative link between lower CNA numbers and lower survival rates. Three studies have investigated the relevance of ploidy status in the onset and progression of PeScc. Masih et al demonstrated a diploid population in verrucous carcinomas (Masih et al. 1992). Ornellas et al performed flow cytometry in 90 cases of PeScc, establishing diploid status in all verrucous cancers, although aneuploid cases varied according to grade: well differentiated (5.5%), moderately differentiated (28.8%) and poorly differentiated (66.6%) (Ornellas et al. 2000). Even so, there were only 3 poorly differentiated tumours in this study, preventing formal analysis of aneuploidy as a

prognostic factor. Nonetheless, there was a tendency towards high DNA index correlating with increased metastatic risk.

Telomerase activity has been briefly studied in penile cancer (Ikeda et al. 2000; Melsheimer et al. 2004; Alves et al. 2001a). Telomerase is an enzyme that adds specific DNA sequence repeats (TTAGGG) to the 3' end of DNA strands in the telomere regions which are found at the ends of eukaryotic chromosomes. The telomeres contain condensed DNA material that confers stability to chromosomes. In cancer, it is proposed that increased telomerase activity can overcome programmed cell death, immortalising the cell and leading to indefinite replicative capacity. This may be linked to increased probability of developing chromosomal alterations and aneuploid populations. Alves et al describes detectable telomerase activity in 85.4% (41/48) of invasive PeScc. Increased levels were also identified in adjacent skin and corpus cavernosal tissue. Similar samples from patients with prostatic carcinoma were telomerase negative. The prognostic significance of these findings requires further clarification, although the changes in surrounding normal tissue suggest a field effect (Alves et al. 2001a).

1.5.5.2. Dysregulation of oncogenes and tumour suppressor genes

It is now accepted that alternative pathways exist to promote tumourigenesis between HPV-dependent and HPV-independent neoplasms. Additional cellular changes are required in HPV negative epithelium to induce full malignant transformation.

1.5.5.2.1. 'p53'

A primary target for researchers evaluating these mechanisms has been the tumour suppressor pathway involving *TP53* gene and its functional protein product, p53. A number of studies have investigated the relationship of p53 expression with HPV infection and prognosis (Lam et al. 1995; Castren et al. 1998; Levi et al. 1998; Lam and Chan 1999; Lopes et al. 2002; Martins et al. 2002; Humbey et al. 2003). It is known that the HPV oncogenic product E6 interferes with this pathway by binding to the oligomerisation region of wild type p53 causing suppression of normal inhibitory function at the G1/S junction of the cell cycle. This leads to uncontrolled cellular proliferation, loss of cellular differentiation and decreased apoptotic control through Bcl-2 and Bax imbalance (Camus et al. 2007). Blanton et al demonstrated an *in vitro* model in which foreskin and ectocervical epithelial cells were infected with retroviral vectors expressing HPV 16 oncogenes (Blanton et al. 1992). Expression of HPV 16 E7 caused persistent proliferation in the suprabasal domain, but no effect on terminal differentiation. E7 expressing cells were positive for p53 but those cells co-expressing E6 and E7 were negative for p53, yet still proliferating suprabasally. The authors concluded that E7-induced suprabasal proliferation is independent of baseline p53 levels. Additionally, recent results have demonstrated that oral keratinocytes infected with E6 deletion mutants *in vitro* showed that increased telomerase activity was closely associated with cell immortalization by BMI-1 and HPV-16 E6, whereas p53 degradation was not (Kim et al. 2007).

The evidence linking p53 expression and presence of HPV DNA in PeScc is contradictory. A study by Lam demonstrated that 100% of HPV infected cells also showed positive p53 staining (Lam et al. 1995). However, several reports have shown

an inverse or negative relationship (Pilotti et al. 1993; Ranki, Lassus, and Niemi 1995; Castren et al. 1998). Castren et al reviewed the expression profile in premalignant conditions (treatment resistant genital warts, bowenoid papulosis, Queyrat's erythroplasia and carcinoma in situ) and compared this to profiles in invasive tumours. The authors concluded that there was no correlation between p53 and HPV status. Over expression of p53 does not indicate a p53 mutation in pre-malignant disease. This infers that *TP53* gene mutations may occur in the later events of male genital carcinogenesis and thus describe a subset of patients with alternate disease progression.

The *TP53* gene is situated on chromosome 17p13 and mutation may lead to either expression of a mutant protein (90%) or absence of protein (10%). Intracellular p53 levels are controlled through a negative feedback loop with its regulatory protein Mdm2. It has been shown that mutant p53 will fail to bind to Mdm2, thus resulting in the accumulation of the oncogenic protein in cells. Ouban et al have proposed that over expression of Mdm2 is important in aberrant p53 down-regulation in PeScc (Ouban et al. 2003).

Studies exploring the expression of p53 in invasive PeScc have shown that overall expression of the protein varies between 40-89%, with expression localised to the periphery of the tumour cell nests in poorly differentiated tumours. No study has shown a correlation with grade or stage of disease. The p53 protein has not been detected in verrucous tumours and shows a difference in expression between histological types (Rubin et al. 2001; Martins et al. 2002) . A strong association between polymorphisms at codon 72 of *TP53* with cancer development and

progression has been shown in several Squamous cell carcinoma models. It is proposed that the arginine (Arg) homozygous genotype presents an increased risk to disease progression in cervical cancer. However, contradictory evidence in other studies refute these data; which has also been reproduced in Ugandan and Italian men with penile cancer (Tornesello et al. 2008). A link between HPV infection and PeScc was confirmed, but this study concluded that the Arg/Arg genotype was not a risk factor in this series. There was no correlation found between the *TP53* polymorphism at codon 72 and the presence of HPV DNA.

The utility of p53 as an independent prognostic marker in patients with penile cancer has been examined by Lopes (Lopes et al. 2002) in 82 patients treated with penile amputation and bilateral lymphadenectomy. The authors were able to demonstrate nuclear accumulation of p53 in 34 of 82 samples analysed (41.5%). The immunoreactivity of p53 was studied alongside other clinicopathological factors, lymph node status and survival. The relationship between p53 expression and HPV infection was also analysed. The results from this study showed that p53 staining was an independent predictor of lymph node metastases on multivariate analysis. Patients with negative p53 had significantly better 5 and 10 year overall survival. Overall outcome was significantly worse if tumours were p53 and HPV DNA positive. Patients who were HPV positive but p53 negative had the best survival figures thus supporting the work performed by Lont. Martins reviewed the outcome in 50 patients and explored the relationship between p53 and proliferating cell nuclear antigen (PCNA) (Martins et al. 2002). The data supported the findings above, but was unable to show that PCNA was of prognostic significance in this disease. Further work is

necessary to help delineate the initiation and progression of these alternate pathways in men with penile cancer.

1.5.5.2.2. 'p21'

Lam and Chan developed their initial findings of p53 expression in PeScc to include p21 expression data in order to investigate the interplay between these two proteins (Lam and Chan 1999). The p21 protein is a primary target for p53 by negatively regulating cell cycle progression through its interaction with cyclin-dependent kinases (CDK) at the G1/S and G2/M checkpoints of the cell cycle. Nuclear staining for p21 protein was achieved in 40% of tumours with suprabasal localisation. This staining was also observed in adjacent dysplastic areas. There was no correlation with grade or stage. There was a strong association between HPV 16 positive cases and positive p21 immunostaining (100%). The authors suggest that in penile cancer p21 expression is controlled by p53 dependent and independent mechanisms.

1.5.5.2.3. 'Bax and Bcl-2'

The complex interplay between the pro-apoptotic protein (Bax), anti-apoptotic protein family (Bcl-2) and p53 dysregulation is well described for various tumours. Recent studies have reviewed this area in penile cancer (Nascimento et al. 2004; Saeed et al. 2005). Both studies revealed an imbalance in Bax/Bcl-2 ratios between benign, pre-malignant and invasive disease. Bcl-2 levels were significantly elevated in low grade disease compared to verrucous cancers, whilst Bax levels were comparable.

1.5.5.2.4. 'p16^{INKA}/cyclin D/Rb'

The p16^{INKA}/cyclin D/Rb pathway is of significant interest in the pathogenesis of penile cancer. This pathway has been shown to be dysregulated in many human carcinomas with inactivation of single or multiple steps within the pathway. The retinoblastoma (Rb) protein is a target for the viral oncoprotein E7, thus its inactivation may contribute to carcinogenic events in HPV-dependent tumours.

This pathway may be disrupted by three independent mechanisms in penile cancer (Ferreux et al. 2003). Analysis of 52 cases of invasive PeScc were scrutinised for p16^{INKA} and BMI-1 using immunohistochemical techniques. HPV 16 E6/E7 mRNA and p16^{INKA} methylation were evaluated using polymerase chain reaction. HPV presence was also compared using in-situ hybridisation. The authors described HPV-dependent and independent mechanisms affecting the normal functioning of this important signalling pathway. These mechanisms include: (1) blocking of Rb function by the E7 oncoprotein with upregulation of p16^{INKA}; (2) silencing of the p16^{INKA} gene by methylation in the absence of HPV infection 15% of cases with p16 over expression; (3) over expression of BMI-1 gene - which targets down-stream factors p16^{INKA} and p14^{ARF} – was also found in 10% of tumours in the absence of HPV infection. The authors postulated that p16^{INKA} may act as a potential prognostic marker and that these results have strong implications on the potential effectiveness of prophylatic HPV vaccines in this tumour, with only a quarter of patients with PeScc potentially benefiting from this treatment directly.

1.5.5.2.5. 'c-ras, myc'

Proto-oncogene activity in penile cancer have been analysed through *c-ras*^{Ha} (Leis et al. 1998) and *myc* (Couturier et al. 1991; Sastre-Garau et al. 2000) mutations and their relationship to HPV involvement. The 21 kDa ras protein has intrinsic GTPase activity, that is regulated by other protein factors, and acts as a focal point for several signal transduction pathways. It is located on the cytoplasmic side of the cell membrane. The codons responsible for inter-protein reactivity are located at codons 32 to 40. Point mutations have been identified that result in constitutive Ras activity. Leis et al examined two cases of PeScc, with one developing late relapse from inguinal metastases in the fifth year (Leis et al. 1998). HPV 18 was isolated in the primary tumour and nodal metastasis which was linked to mutations in *TP53* and *c-ras*^{Ha}. Whilst HPV infection may be involved in early carcinogenesis, mutation in *ras* genes occur later and are linked to disease progression. Two studies (Couturier et al. 1991; Sastre-Garau et al. 2000) from the same French institution investigated the pattern of genomic integration of HPV DNA in a small number of cases of penile cancer. They highlighted that the integration of HPV types 16 and 18 was localised to sites containing *c-myc* (8q24.1) and *n-myc* (2p24) proto-oncogenes. They also demonstrated genetic rearrangements in 3 out of 4 cases. Unfortunately, only one cell line was derived from a primary PeScc; however, these results warrant further validation.

1.5.5.3. Cell cycle control

The importance of cell cycle regulation is a cornerstone for normal cellular proliferation and genomic stability. As alluded to in previous sections, the cell cycle is under the tight influence of numerous signalling pathways involving different proto-

oncogenes and tumour suppressor genes. The influence of p53 on cell cycle control and how the loss of the normal genotype can promote carcinogenic events has been previously described. Alternative factors involved in cell cycle regulation investigated in penile cancer include PCNA, which was shown not to have any prognostic benefit. In addition, the nuclear protein Ki67 which helps to identify the growth fraction within tumours has also been investigated.

Ki67 is expressed in all phases of the cell cycle (G1/S/G2/M) but is absent from cells which have exited the cell cycle to reside in the G0 state (senescence, terminal differentiation or quiescence). This biological attribute allows Ki67 to identify the cells which are actively cycling. In normal squamous epithelium this growth fraction with positive immunohistochemical staining is restricted to the basal compartment. Medina et al investigated the localisation of Ki67 protein in verrucous tumours of the penis and demonstrated that proliferative activity was detected at the growing edge of the tumour (Medina, Valero, and Martinez Igarzabal 1999). This reflects the well differentiated nature of these tumours and the hypothesis that relative increased cellular activity is at the invasive edge of PeScc. p53 staining was negative in this study, in keeping with previous reports in verrucous cancers. Berdjis et al performed an analysis in 44 patients with penile cancer studying the relationship of Ki67 expression to grade, stage and nodal status (Berdjis et al. 2005). The mean labelling index (LI) was 40.5% (6.4 to 93%) for all tumours analysed with the wide range of expression reflecting the strong correlation of Ki67 staining to tumour grade ($p<0.005$). There was no significant association with tumour stage or lymph node metastases ($p=0.07$). No survival data was available, so it was not possible to comment on the potential of Ki67 as a prognostic marker in this study. A recent

report by Gentile et al investigated HPV, p53 and Ki67 associations in 11 PeScc specimens; with ten out of eleven tumours demonstrating positive expression for Ki67 and p53 proteins. HPV positive cases also had high levels of p53 (80%) and Ki67 (70%) (Gentile et al. 2006).

1.5.5.4. Cyclo-oxygenase pathway

Prostaglandin production via the cyclo-oxygenase pathway has been implicated as an important regulator and effector influencing multiple events in carcinogenesis and tumour invasion. Prostaglandin E2 (PGE2) has been identified as a bioactive product shown to stimulate cell proliferation, inhibit apoptosis, modulate angiogenesis, cell to cell signalling and suppress immune surveillance. Induction of the pathway has been linked to several carcinogenic promoters including polycyclic aromatic hydrocarbons produced by smoking. The pathway is also regulated by a number of molecules including growth factors, glucocorticoids, cytokines and inflammatory proteins.

Two distinct cyclo-oxygenase isoforms (Cox-1 and Cox-2) exist which convert arachidonic acid into the intermediaries PGG2 and PGH2, both of which undergo further enzymatic change to produce the final PG products. Prostaglandin E synthase-1 is responsible for the production of PGE2. In general, Cox-1 (“house-keeping”) is constitutively expressed, whilst Cox-2 (“inducible”) is not expressed in normal epithelium but induced through the factors described above. Over expression of Cox-2 has been detected in various pre-malignant and malignant tissues; including oesophageal, lung and head/neck Scc (Shamma et al. 2000; Kim et al. 2002; Chang et al. 2004).

Golijanin et al have investigated the expression of Cox-2 and PGE synthase-1 in a small cohort of patients with PeScc (Golijanin et al. 2004). The authors clearly demonstrated an up-regulation of both factors in dysplastic and invasive cancer specimens with granular cytoplasmic staining compared to no expression in normal epithelium. Due to the small numbers of specimens analysed (n=7), it was not possible to draw definitive conclusions with respect to the potential utility of these factors as prognostic markers. Larger scale studies expanding on the interesting findings from this study may identify Cox-2 and associated proteins as both novel diagnostic, prognostic and therapeutic targets for future clinical trials.

1.5.5.5. Invasion, metastasis and angiogenesis

These events have been well described as essential in the progression of confined, local disease to stromal and lymphovascular invasion (Bissell and Radisky 2001). The first study analysing this important area in penile cancer was recently published, analysing E-cadherin (involved in intercell adhesion) and matrix metalloproteinases (MMP-2 and MMP-9) (involved in breakdown of extracellular matrix) in 125 patients with PeScc (Campos et al. 2006). The authors provided good evidence that low E-cadherin immunoreactivity is associated with a greater risk of lymph node metastases and high MMP-9 expression was an independent risk factor for disease recurrence. These markers should be analysed further in prospective, multi-institutional studies.

1.5.5.6. Squamous cell carcinoma antigen (Scc-Ag)

Two genes *(SCC Ag-1* and *SCC Ag-2)* have been identified that have highly homologous protein products which have been classified as serine protease inhibitors. The utility of these serological markers have been evaluated in several studies

involving penile cancer (Wishnow, Johnson, and Fritsche 1989; Wishnow, Johnson, and Fritsche 1990; Laniado et al. 2003) revealing that they may provide information for detecting lymph node metastases in penile cancer, either at diagnosis (sensitivity 57%; specificity 100%) or for patients entered into surveillance programs. Larger studies, perhaps focusing on Scc-Ag transcripts, are needed to fully evaluate the impact of this marker on clinical management in these patients.

1.6. Chapter Summary

Penile cancer is a rare malignancy associated with poor survival outcomes for patients with advanced disease states. Improving Outcomes in Urological Cancers Guidance (IOG) was released in 2002 (National Institute for Clinical Excellence 2002), and may have had an effect on mortality as more appropriate treatment is offered, and multi-disciplinary teams (MDTs) have been formed to discuss treatment options. However, identifying patients who will benefit from aggressive therapeutic strategies remains problematic. The delineation of novel effective molecular targets capable of delivering powerful prognostic information as well as defining new therapeutic targets in this clinical setting is challenging. There is clear evidence of a fascinating, mixed heritage for tumourigenesis in penile cancer involving virological, inflammatory and genetic factors; all of which may act on various molecular pathways to induce and promote cancer states.

In light of the important biological, prognostic, and therapeutic implications of RLFs as cell cycle regulators in normal cell proliferation and tumourigenesis, I have investigated their role in penile carcinoma. I have evaluated the multiparameter analysis of Mcm2, geminin, and Ki67 to study the cell cycle kinetics of this tumour

type *in vivo* and how deregulation of the replication licensing pathway may be linked to acquisition of aneuploidy.

Using this panel of cell cycle biomarkers, I aim to identify key "proliferation signatures" that reflect aggressive cell cycle phenotypes linked to poorer clinical outcomes. Integration of these biomarkers with conventional clinicopathologic parameters in a novel predictive model has the potential to facilitate identification of those patients most likely to benefit from radical surgical and chemotherapeutic interventions.

My findings will provide new insights into the biological mechanisms involved in tumour progression of penile carcinoma and how these novel biomarkers of growth might be exploited to predict the *in vivo* behaviour of this rare tumour type.

CHAPTER TWO

MATERIALS & METHODS

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CHAPTER TWO

MATERIALS & METHODS

2.1. General

2.1.1. Chemicals and reagents

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless specifically referenced by source.

20x TRIS BUFFERED SALINE

60.6g	Trizma Hydrochloride
13.9g	Trizma base
87.6g	Sodium chloride
500ml	Distilled water

5X PHOSPHATE BUFFERED SALINE (TABLETS)

42.5g	Sodium chloride
32.4g	Disodium hydrogen phosphate
0.78g	Sodium dihydrogen phosphate

Make up to 1 litre with distilled water; pH 7.3

4X LAEMLLI SAMPLE BUFFER

33 mM	Tris-Cl (pH 6.8)
3.3% (w/v)	SDS
30% (v/v)	Glycerol
17% (v/v)	β-mercaptoethanol
0.02% (w/v)	Bromophenol blue

5X TRIS-GLYCINE ELECTROPHORESIS BUFFER

125 Mm	Tris-base
1.25 M	Glycine
0.5% (w/v)	SDS

TRANSFER BUFFER

0.3 M	Tris-base
10 mM	CAPS
0.02% (w/v)	SDS
10% (v/v)	Methanol

GEL LOADING BUFFER

10% (v/v)	Glycerol
0.025% (w/v)	Bromophenol blue

TRIS-EDTA BUFFER

10 mM	Tris-HCl
1 mM	EDTA (pH to 8.0)

FIXATION BUFFER

4%	Para-4-aldehyde
0.1%	Glutaraldehyde

2% BSA/TBS SOLUTION

100mg	BSA
5ml	TBS solution

CITRATE BUFFER (0.1 M)

80g	Sodium citrate
10l	Distilled water (pH to 6.0)

PERMEABILISATION BUFFER

0.1%	Triton X-100
0.02%	SDS

LYSIS BUFFER

50 mM	Tris-Cl (pH 7.5)
150 mM	NaCl
20 mM	EDTA
0.5% (v/v)	NPO4

2.2. Acquisition of clinical data

2.2.1 Clinical database

Clinical and pathologic information was retrieved for 141 patients with squamous cell carcinoma of the penis stored on the secure hospital databases. SNOMED searches included the terms: *penile carcinoma, urethral carcinoma, circumcision and penectomy* for the period 1988-2007. All patients had been treated within the North Thames Cancer Network for penile cancer. The following clinical information was sourced directly from patients' hospital medical records: date of birth, date of diagnosis, clinical stage, site, evidence of BXO, smoking status, primary and secondary surgery, date of last follow-up, and date and cause of death (Appendix A). Information was collated and stored in a clinical database held in the Department of Urology (University College London Hospital, London, UK).

Patients who entered into surveillance programs without lymph node surgery were classified as negative after 2 years without disease presentation. Twelve patients with carcinoma in situ were removed from any formal survival analyses and 11 patients were lost to follow-up. Therefore, 118 patients were included in the long-term follow-up survival study. The median follow up time was 20 months (range, 0.8-162.4 months). Mean survival time amongst those who had died was 21.4 months (SD=25.6 months, range 1-138 months).

2.2.2 Tissue specimens

Ethical approval was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (Appendix B). Histologic specimens were reviewed by a uro-oncology pathologist at diagnosis. Paraffin wax–embedded tissue specimens were

retrieved from the pathology archives for all patients. Excised tumours were histologically staged using the revised tumour-node-metastasis (TNM) system criteria 2002 (Sobin LH and Wittekind C 2002). Pathologic variables of the primary tumour included: grade, local stage, subtype, extent (unifocal/multifocal), tumour size, depth of invasion, and lymphovascular invasion. All pathologic parameters were recorded by a specialist uro-oncology pathologist and independently reviewed by a second pathologist. Tumour grade was defined using Broders' classification (Broders AC. 1921): well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3). Tumour size was defined as the maximal dimension and depth of invasion measured from adjacent normal epithelium to the deepest invasive point. Lymphovascular invasion was determined microscopically and confirmed using antibodies against endothelial markers CD33 and CD34. Lymph node status was confirmed following pathologic review of inguinal and pelvic lymph node specimens attained through prophylactic or delayed lymphadenectomy.

2.3. Immunological techniques

2.3.1. Antibodies

Affinity-purified rabbit polyclonal antibody against full-length *hs*geminin and was produced in the host laboratory as described (Wharton et al. 2004). To summarise; pET15b-human (hs)Geminin was expressed in Escherichia coli strain BL21(De3) and purified by Ni-NTA metal affinity chromatography following the manufacturers' instructions (Qiagen, Crawley, UK). Recombinant hsGeminin was further purified using an (HPLC) Hiload Q sepharose 16/10 column in NaPi buffer and eluted with varying concentrations of 1 M NaCl. Four rabbits were injected with 125 mg of purified hsGeminin protein, and received three boost injections over a period of 80

days following a standard protocol (Eurogentech, Seraing, Belgium). The sera were collected and affinity-purified on a CNBr column against 10 mg of recombinant hsGeminin protein, eluted with 0.1 M glycine (pH 2.5) and dialysed into PBS, 1% BSA and 0.1% sodium azide. An equal volume of sterile glycerol was added and affinity-purified polyclonal antibodies G92 and G95 were stored at -20^{o}C. Antibody purification was quality controlled by SDS–PAGE for purity and by ELISA. Specificity of the affinity-purified antibodies was demonstrated by immunoblotting of cell lysates and by quenching all immunohistochemical staining after incubating antibodies diluted at working concentrations with equal or less than molar amount of recombinant *hs*Geminin protein for 1 h prior to a standard immunohistochemical staining protocol. The following antibodies were sourced commercially: Ki-67 monoclonal Ab (clone MIB-1); CD31 monoclonal Ab (clone JC70A) & CD34 monoclonal Ab (clone QBEnd10) were obtained from DAKO (Glostrup, Denmark), Mcm2 monoclonal Ab (clone 46) from BD Transduction Laboratories (Lexington, KY) and Cdt1 monoclonal Ab (clone 14676) from Abcam (Cambridge, UK). The specificity of Ki67 and Mcm2 monoclonal antibody has been extensively studied and validated in previous studies (Dudderidge et al. 2005; Shetty et al. 2005; Kulkarni et al. 2007; Loddo et al. 2009).

Antibody	Immunogen	Source	WB condition	IHC condition
Actin MAb (clone AC-74)	β-actin N-terminus	*Sigma A5316*	5% milk 1:2500	-
CD31 MAb (clone JC70A)	CD31 N-terminus	*DAKO* M7240	-	1:20
CD34 MAb (clone QBEnd10)	CD34 N-terminus	*DAKO* M7240	-	1:50
Cdt1 MAb	Cdt1 C-terminus 533-546aa	*Abcam* Ab14676	5% milk; 0.1% tween 1:300	-
Geminin PAb (#2095)	geminin Full-length	*In-house*	10% milk 1% tween 1:1000	1:1500 dilution
Ki67 MAb (clone MIB-1)	Ki67 1002 bp peptide	*DAKO* M7240	-	1:70 dilution
Mcm2 MAb (clone46)	Mcm2 C-terminus 725-888aa	*BD Labs* BM28	5% milk 0.1% tween 1:2000	1:1000 dilution

Table 2.1 – Antibody specifications

2.3.2. Immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using pre-cast Novex 4-20% Tris-glycine gels with the XCell Surelock™ mini-cell electrophoresis system (Invitrogen). Protein samples were mixed with 4x laemlli buffer, heated to 90^{o}C for 3 minutes and pulse centrifuged. 50 µg protein was loaded per well according to associated Bradford assay calculations. Samples were run in 1x Tris-glycine running buffer at 125 mV for approximately 90 minutes. Protein migration was confirmed using Ponceau red staining. Protein transfer from polyacrylamide gels to nitrocellulose membranes (Amersham Biosciences Buckinghamshire, UK) was achieved using semi-dry electroblotting in transfer buffer. Non-specific binding was blocked siting the membranes in 5% skimmed milk powder, 0.1% Tween-20 and PBS overnight at 4^{o}C. Primary antibodies were introduced in the following dilutions and conditions and incubated for 2 hours at RT: Mcm2 (1/2000; 5% milk; 0.1% tween),

geminin (1/1000; 10% milk; 1% tween), Cdt1 (1/300; 5% milk; 0.1% tween) and Actin control. Membranes were washed in PBS/0.1% Tween-20 solution for 5 minutes and repeated for 6 washes. Secondary antibodies were applied and incubated in the above conditions for 1 hour at RT. Finally, membranes were washed again for 6 washes and immunoreactive bands visualised on photographic film (Kodak) by enhanced chemiluminescence (ECL, Amersham Biosciences).

2.3.3. Immunohistochemistry

Antibodies used were those described previously (2.3.1). Archival formalin-fixed, paraffin-embedded tissues were retrieved as described (2.2.2). Archival formalin-fixed, paraffin-embedded tissue (PWET) obtained at initial diagnosis was available for all patients and for each specimen a block was chosen that contained a representative sample of invasive tumour. Consecutive serial sections cut from each PWET block were used for immunohistochemistry. Three µm tissue sections were cut onto Superfrost Plus slides (Visions Biosystems, UK), dewaxed in xylene and rehydrated through graded alcohol to water. For antigen retrieval, slides were pressure cooked in 0.1 mol/L citrate buffer (pH 6.0) at 103kPa for 2.5 min. Tissue sections were immunostained using the Bond^{TM} Polymer Define Detection kit and Bond^{TM}-x automated system (Vision Biosystems, Newcastle Upon Tyne, UK) according to the manufacturer's instructions. The following protocol was used for automated staining; peroxidase activity was quenched using peroxidase blocking solution for 10 min. Sections were washed and incubated with protein block for 10 minutes. Following washing, primary antibodies were applied to sections at the dilutions stated (Table 2.1.) and incubated for 40 min. Sections were washed and post-primary was applied for 30 min followed by washing and addition of polymer for a further 30 min. After

washing, immunostaining was developed using 3,3 diaminobenzidine tetrahydrochloride (DAB) for 10 min followed by Bond^{TM} DAB enhancer (AR9432, Vision Biosystems). After washing in dH_20, sections were counterstained with Meyer's haematoxylin for 5 min, differentiated in 1% acid alcohol, dehydrated through water to alcohol and cleared in xylene. Coverslips were applied with Pertex mounting medium (CellPath Ltd, Newtown Powys, UK). Incubation without the primary antibody was used as a negative control and colonic epithelial sections were used as positive controls.

2.3.4. Protein expression profiling

Protein expression analysis was done by determining the labelling index (LI) of the markers in each tumour, as previously described (Meng et al. 2001; Shetty et al. 2005; Dudderidge et al. 2005; Kulkarni et al. 2007; Loddo et al. 2009). Consecutive serial sections cut from the same formalin-fixed, paraffin-embedded tissue block were used to stain for the markers. Slides were evaluated at ×100 magnification to select the advancing edge of the tumour. Selected areas at the advancing edge plus three to five adjacent fields perpendicular to the advancing front moving progressively towards the centre of the tumour were image captured at ×400 magnification with a charge-coupled device camera and AnalySIS image analysis software (SIS). Images were subsequently printed for quantitative analysis, which was undertaken with the observer unaware of the clinicopathologic variables. Both positive and negative cells within the field were counted and any stromal or inflammatory cells were excluded. A minimum of 500 cells was counted for each case. The LI was calculated using the following formula: LI = number of positive cells/total number of cells × 100. Normal foreskin and colon specimens were used as external controls. Normal adjacent

epithelium acted as an additional internal control. Reassessment of 10 randomly selected cases by an independent assessor showed high levels of agreement.

2.4. Cell biology techniques

2.4.1. Cell culture and synchronisation

HeLa S3 tissue culture cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% foetal calf serum (FCS, Gibco-BRL). SKOV3 tissue culture cells were maintained in McCoy's 5A medium (Gibco-BRL) supplemented with 15% FCS (Gibco-BRL). For both cell lines growth media also contained 100 U/ml penicillin and 0.1 mg/ml streptomycin. HeLa cell cycle synchronisation was performed essentially as described (Krude *et al* 1997). Briefly, for preparation of cells in G1/S, cells were synchronised in very early S phase by two sequential 25 h blocks in 2.5 mM thymidine separated by a 12 h interval without thymidine (Rao and Johnson 1970). For preparation of cells in G2/M phase, cells were released from the second thymidine block for 3 h, followed by adding 40 ng/ml nocodazole for an additional 12 h to arrest them in mitosis (Johnson *et al* 1993). For SKOV-3 cell cycle synchronisation, cells were synchronised in very early S phase by one 25 h block in 2.5 mM thymidine. Cells were released from the thymidine block for 3 h, followed by adding 40 ng/ml nocodazole for an additional 12 h to arrest them in mitosis.

2.4.2. Preparation of total cell extracts

HeLa S3 and SKOV3 cells (asynchronous and synchronised time points) were harvested by treatment with trypsin, washed in PBS, and resuspended in lysis buffer

at $5x10^5$ cells/ml. After incubation on ice for 30 minutes, the lysate was clarified by high speed centrifugation (13,000g, 15 min, 4°C) and stored at -80°C.

2.4.3. *Bivariate flow cytometric analysis*

For cell cycle analysis of DNA content cell samples were fixed in 80% ethanol for a minimum of 12 h. After fixation, cells were washed twice in dPBS and incubated for 20 min with 0.5 ml PI (20 µg/ml) and RNase A (50 µg/ml) (Helmstetter *et al* 2003) and 0.5 ml dPBS at RT. To prevent clumping of cells and loss of sample, 100 µl of 1% BSA was added at each step and unautoclaved pipette tips were used. Analyses of DNA content were performed using a FACSCalibur flow cytometer (BD Biosciences). Cell doublets were excluded where necessary by gating on a dot plot of the width vs the area of DNA fluorescence intensity (Erlanson and Landberg 1998). In most samples, 10^4 cells were examined and data were analysed using CellQuest™ software (BD Biosciences) and WinMDI (V 2.8).

2.5. *DNA image cytometric analysis*

For each case, a 40µm section of formalin-fixed, paraffin-embedded tissue obtained from the same block as that assessed by immunohistochemistry was used to prepare a suspension of nuclei. PWET sections were placed in 50ml falcon tubes and dewaxed in xylene followed by rehydration through graded alcohol to water and finally suspended in cold PBS. The supernatant was aspirated and excluded between solutions. Protease XXIV (2ml) was added to the specimens and incubated for 2h at 37°C in a shaker water bath. Chilled PBS (3ml) was added to quench the enzymatic digestion and the samples were filtered through nylon mesh filters. Samples were centrifuged at 1500 rpm for 5 minutes and the supernatant discarded. The pellet was

re-suspended in 3ml fresh PBS then cytospun in 100 and 200µl aliquots at 1500 rpm for 5 minutes to prepare a monolayer of nuclei on Superfrost glass slides (Visions Biosystems UK). The density of the nuclear preparation on the slide was checked under a light microscope using toludine blue stain and an adjusted volume of the suspension was cytospun if correction of the density was required. The monolayer preparations were air dried and fixed overnight in 4% formaldehyde. After washing in distilled water, slides were incubated in 5 mol/L HCl for 1h at room temperature for hydrolysis. Slides were then rinsed in distilled water and incubated in Feulgen-Schiff's solution for 2h in the dark. Finally, the slides were washed in running tap water for 10 minutes, dehydrated in increasing alcohol gradient, cleared in xylene, and coverslipped.

The Fairfield DNA Ploidy System (Fairfield Imaging Ltd, Nottingham, UK) was used for image processing, analysis and classification (Figure 2.1). Monolayers were analysed with the use of a Zeiss Axioplan II microscope (Zeiss, Oberkochen, Germany) that was equipped with a 546nm green filter (plus X40 lens and 0.65 objective). A modified stage was used linked to a coordinate computer model (H152V2, Prior Scientific Instruments, Fulbourn, Cambridge, UK). The microscope was also equipped with a single-chip digital camera (model C4742-95, Hamamatsu Photonics, Japan.). The final magnification was x1600 at an estimated resolution of 170nm (0.2µm) per pixel; the visual field measured 1024 by 1024 pixels and had a 10 bit resolution (1024 gray levels). The nuclei of at least 1000 cells were collated and the information stored as a ‘gallery’ in a computerised folder. Lymphocytes and plasma cells were included as internal controls, and sections of high-grade bladder

tumour and normal foreskin tissue were analysed as external controls for the aneuploid and diploid populations, respectively.

Histograms were classified according to the following previously published criteria (Haroske G et al. 1997 ESACP consensus report). The tumour was classified as diploid if only one G_0-G_1 peak (2c) was present, the number of nuclei in the G_2 (4c) peak did not exceed 10% of the total number of nuclei, and the number of nuclei with a DNA content exceeding 5c did not exceed 1%. A tumour was defined as tetraploid when a peak in the 4c position was present together with a peak in the 8c position or the fraction of nuclei in the 4c region exceeded 10% of the total number of nuclei. A tumour was defined as polyploid when a peak in the 8c position was present together with a peak in the 16c position. The tumour was defined as aneuploid when noneuploid peaks were present or the number of nuclei with a DNA content exceeding 5c/9c, not representing euploid populations, exceeded 1%. The histograms were classified by two independent assessors with a high level of agreement and without knowledge of the clinicopathologic variables. For the purposes of statistical analysis, tetraploid and polyploid tumours were grouped together with aneuploid tumours (technical figure 2.1).

Figure 2.1

2.6. Statistical analysis

Relationships between biomarker expression and other factors were assessed using the Mann-Whitney U, Kruskal-Wallis, and Jonckheere-Terpstra tests. Data were summarized as the median value and interquartile range of LIs observed across the cohort. Multivariable analyses for lymph node status were carried out in three steps using logistic regression: (a) all factors were assessed separately and those with $P < 0.05$ were retained, (b) remaining pathologic and biomarker factors were entered into two separate models and backward elimination was applied with $P = 0.05$, and (c) remaining factors were entered into a single model and backward elimination was applied to produce a final model. Multivariable analyses for overall survival were carried out in a similar fashion using Cox's proportional hazards model. The discriminatory ability of this model was quantified using Harrell's c-index (Harrel 2001), which is analogous to the receiver operating characteristic area and gives the probability that two randomly selected patients have concordant predictions and survival times. The c-index takes values between 0.5 (random predictions) and 1 (perfect concordance). Patients were divided into tertile model-based risk groups and labeled as low, medium, and high risk for disease progression. Patients with incomplete data were excluded from multivariable analyses. All tests were two sided and used a significance level of 0.05 with 95% confidence intervals (95% CI), and no allowances were made for multiple hypothesis testing. All analyses were done using Stata 10 for Windows (StataCorp).

2.7. Computing and data storage

Clinical information, including patients' personal details, was stored in dual electronic database formats (MSExcel & MSAccess Microsoft Corporation, USA). This

information was made available to the National UK Andrology Database, a SSL encrypted web based real-time database (written and developed by Mr. Nim Christopher) and linked to the British Association of Urological Surgeons (BAUS) cancer registry. All images were generated using Adobe Illustrator and Photoshop version 7.0 (Adobe Systems Inc, USA) using only standard contrast and brightness adjustment functions. The text of this thesis was constructed in MSWord (Microsoft Corporation, USA).

CHAPTER THREE

INTEGRATED STAGING SYSTEMS IN PENILE CANCER: DEVELOPING A PREDICTIVE MODEL USING TUMOUR PLOIDY INFORMATION

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(b) Normal squamous epithelium

(c) Squamous hyperplasia

(d) Balanitis Xerotica Obliterans (BXO)

(d) Carcinoma in situ (Cis)

3.2.3.2 Histology of malignant squamous lesions

(k) Squamous cell carcinoma, usual type (NOS)

(l) Verrucous carcinoma

(m) Warty (condylomatous) carcinoma

(n) Paillary carcinoma

(o) Basaloid carcinoma

(p) Sarcomatoid carcinoma

(q) Carcinoma cuniculatum

(r) Pseudohyperplastic carcinoma

(s) Adenosquamous carcinoma

(t) Acantholytic carcinoma

3.2.3.3 Grading

3.2.3.4 Staging

3.2.3.5 Measurements

CHAPTER THREE

INTEGRATED STAGING SYSTEMS IN PENILE CANCER: DEVELOPING A PREDICTIVE MODEL USING TUMOUR PLOIDY INFORMATION

3.1. Introduction

Therapeutic algorithms in the management of patients with squamous cell carcinoma of the penis are currently reliant on risk stratification based on clinical information and primary tumour characteristics (Solsona et al. 2004; Ficarra et al. 2006; Kattan et al. 2006a). In patients with localized disease, the most relevant prognostic feature is the identification of clinical and/or pathologic factors able to predict the presence of metastatic disease within regional lymph nodes. Lymph node involvement is the strongest predictor of survival in this patient group with data supporting a survival benefit following radical surgical resection of involved nodal tissue (Ravi 1993b). Patients with palpable inguinal lymphadenopathy (*clinically positive; cN+*) have a high probability of habouring loco-regional disease as reported in several large case series with early lympadenectomy advocated. However, some patients may present with reactive lymphadenopathy secondary to concomitant infection thus confusing the clinical picture. Conversely, in patients with impalpable inguinal lymph nodes (*clinically negative; cN-*); approximately 20% of patients will harbour micrometastases who may be disadvantaged if placed into surveillance programmes incorrectly (Lont et al. 2003b; Lont et al. 2003a; Kroon et al. 2005).

The diagnostic and therapeutic dilemma is further complicated given the significant morbidity and mortality associated with these surgical techniques which limit their routine implementation in all patients (Ravi 1993a; Horenblas 2001). Finally, the role of adjuvant and neoadjuvant chemotherapy is poorly understood with limited level A/B evidence to support any single agent or regime (Pizzocaro and Piva 1988; Yumura et al. 2007; Bermejo et al. 2007; Leijte et al. 2007). Likewise, current chemotherapeutic regimes in disseminated disease are linked to poor clinical outcomes and high levels of toxicity (Protzel and Hakenberg 2009). Therefore, patient identification and selection for aggressive surgical approaches, as well as inclusion for neoadjuvant and adjuvant chemotherapeutic programmes remains a challenging problem in the management of these patients.

Since the development of this diagnostic and treatment paradigm, numerous studies have attempted to identify clinical and pathological factors that may help to predict lymph node involvement; as a surrogate marker of survival and outcome. This led to the publication of guidelines developed through the European Association of Urologists (EAU) consensus group (Solsona et al. 2004), who devised and prospectively validated the association of local tumour stage and grade as a predictive factor for occult lymph node metastasis in patients with clinically negative (cN-) inguinal lymph nodes.

Subsequent studies have further evaluated the impact of these standard pathological factors in conjunction with newer pathological parameters. Important factors identified with strong associations with lymph node metastases include: depth of invasion, size of tumour, tumour thickness, lymphovascular invasion and tumour

growth patterns (Ornellas et al. 1994; Cubilla et al. 2001a; Cubilla et al. 2001b; Lopes et al. 1996; Slaton et al. 2001).

The lack of diagnostic discriminatory power related to current imaging modalities (ultrasound, computed tomography and magnetic resonance imaging) to accurately delineate inguinal and pelvic lymphatic involvement (Horenblas et al. 1991; Rubben and Lutzeyer 1981; Maiche 1993; Scher et al. 2005; Mueller-Lisse et al. 2008) has promoted further development of new nomograms predictive of pathological inguinal lymph node involvement and cancer specific survival in patients with PeScc (Ficarra et al. 2006; Kattan et al. 2006a). It is hoped that these new integrated staging systems will help to diminish the number of unnecessary inguinal and pelvic lymphadenectomie. These prognostic tools await crucial prospective application to fully understand their clinical utility in this patient population.

In order to determine the importance of clinicopathological features of squamous cell carcinoma of the penis in the United Kingdom, I sought to analyse a large, single institution, retrospective review of archival penile carcinoma specimens. The histopathological information has been linked to clinical data in order to validate this database with respect to clinical and demographic information against published case series. Subsequently, I have studied the DNA content of these tumours using image cytometry and correlated this variable against known, standard pathological factors. Finally, I have evaluated the impact of aneuploidy as a novel predictive factor for loco-regional lymph node metastases in men with PeScc.

3.2. Materials and Methods

For Clinical data (2.1.1.2), Antibodies (2.2.1), Immunohistochemistry (2.2.5), DNA ploidy analysis: image-based cytometry (2.2.6), Statistical methods (2.2.7.) refer to Chapter Two. All histological techniques were performed as outlined in the review by Mikuz -'Handling and pathology reporting for circumcision and penectomy specimens' (Mikuz et al. 2004). Sections are highlighted for clarity and to reference the review process undertaken for all archival specimens.

3.2.1. Patient inclusion

Clinical and pathological information was retrieved for 141 patients with squamous cell carcinoma of the penis stored on the secure hospital databases. SNOMED searches included the terms: penile carcinoma, urethral carcinoma, circumcision and penectomy for the period 1988-2004. Patients were also recruited prospectively though the North Thames Cancer Network for penile cancer referrals from 2004 until the end of the study. Archival haematoxylin-eosin stained slides were reviewed and the quality of the paraffin wax embedded samples assessed. Representative tumour sections were chosen and processed for immunohistochemistry as described in section 2.2.6. Primary antibodies were added at dilutions CD31 (1 in 50; Rat Anti-Mouse CD31 (PECAM-1); BD Pharmingen) and CD34 (1 in 80; My10, Mouse anti-Human; Becton Dickinson) in order to assess for the presence of invasion by tumour cells into vascular spaces. All specimens were reviewed by a single pathologist specialising in urogenital pathology.

3.2.2. Specimen processing & preparation

Tissue specimens were handled, fixed and mounted into paraffin wax embedded blocks as previously described. Macroscopic findings were recorded in all incidences. Specific attention was paid to specimen orientation with longitudinal serial sectioning of circumcision specimens. Penectomy specimens were bisected longitudinally with reference to the urethra. Histology sections of the tumour, foreskin, transverse section through the shaft and longitudinal glans sections were required and the resection margin noted (Figure 3.1).

Lymph nodes without overt metastasis greater than 5 mm were cut through the hilus in 2 mm thick parallel slices. Lymph nodes smaller than 5 mm along the longitudinal axis were entirely processed. Negative lymph nodes are entirely processed (steps of 250 μm).

3.2.3. *Pathology reporting*

Pathology reports were generated with reference to the publication by Mikuz (Mikuz et al. 2004) and external validation regarding general histopathology, penile carcinoma subtypes including pathology and outcomes sourced from World Health Organization of Tumours – tumours of urinary system and male genital organs (Eble J.N et al. 2004)**.**

3.2.3.1. *Histology of benign & pre-malignant squamous lesions of the penis*

(a) Normal squamous epithelium

Control tissues sections were retrieved from the archive involving penile epithelium (foreskin +/- glans). Normal epithelial architecture was confirmed such that the

basement membrane and lamina propria were intact with normal cellular proliferation and maturation from basal layers to apical terminally differentiated keratinocytes. Levels of keratin production were noted. Areas of normal epithelium were also assessed as internal controls in all cases with atypical or neoplastic changes.

(b) Squamous hyperplasia

Microscopic findings consisted of elongation, widening of the rete ridges and irregular thickening of the Malpighian layer of rete ridges (acanthosis), hyperkeratosis, and chronic inflammation in dermis. Inflammatory reactions within the dermis consisted of lymphocytes and a small number of plasma cells. Invasive malignancy was carefully excluded in all cases.

(c) Balanitis Xerotica Obliterans (BXO)

Histologically, these lesions demonstrated an atrophic epidermis with the loss of the rete pegs and homogenization of collagen in the upper third, combined with a zone of lymphocytic and histiocytic infiltration. These changes resemble the lesions of lichen sclerosis et atrophicus observed elsewhere in the body. The term is used interchangeably with male genital lichen sclerosus (MGLS).

(e) Carcinoma in situ (Cis)

As seen with cases of intraepithelial neoplasia observed elsewhere, these pre-invasive lesions were characterised by atypical hyperplastic cells showing disorientation, vacuolation, multiple hyperchromatic nuclei and mitotic figures at all

Figure 3.1

Figure 3.1

Figure 3.2

levels. The epithelial rete extended into the submucosa and appeared elongated and bulbous. The submucosa showed capillary proliferation and ectasia with a surrounding inflammatory infiltrate, usually rich in plasma cells. These lesions are usually termed erythroplasia of Queryat if involving the glans/shaft penis or prepuce and Bowen's disease if involving the remainder of the genitalia or perineal skin.

3.2.3.2. Histology of squamous cell carcinoma of the penis

The majority of penile cancers are primary, squamous cell carcinomas (PeScc) originating in the epithelium covering the glans, coronal sulcus, and foreskin. Several histologic subtypes have been described, each with distinctive clinicopathologic and outcome features. Penile verruciform tumours encompass verrucous, warty (condylomatous), and papillary carcinomas. As a group, verruciform tumours are low grade, with low metastatic and mortality rates. In contrast, basaloid and sarcomatoid carcinomas are among the most aggressive penile tumours. Other PeScc variants, such as carcinoma cuniculatum and pseudohyperplastic, adenosquamous and acantholytic carcinomas are rare.

(a) Squamous cell carcinoma, usual type (NOS)

PeScc of usual type (NOS) accounts for 48%-65% of penile carcinomas. Macroscopically, they range from white-to-grey, irregular exophytic to reddish flat and ulcerated endophytic masses. Microscopically, the tumours vary from well-differentiated keratinizing tumours to solid anaplastic carcinomas with scant keratinization. Grading criteria have recently been published and are discussed in section 3.2.3.2. Most tumours are highly keratinized with moderate differentiation. Poorly differentiated carcinomas can have variable amounts of spindle cell, giant cell,

solid, acantholytic, clear cell, small cell, warty, basaloid, or glandular components. PeScc (NOS) has a recurrence rate of 28% and lymph node metastases appear in 28%-39% of cases. The mortality rate is 20%-38% with a 10-year survival rate of 78%.

(b) Verrucous carcinoma

Verrucous carcinoma represents 3%-8% of penile carcinomas. It is slow-growing and extremely well-differentiated, with a papillomatous appearance and broadly based limits between the tumour and stroma. Verrucous PeScc is consistently HPV negative. It can be confused with other tumours, such as giant condylomas and papillary and warty carcinomas. The tumour can be locally aggressive but it is biologically benign with negligible threat for metastatic disease. Macroscopically, it exhibits a cobblestone to filiform surface and rarely invades beyond the lamina propria, superficial dartos, or corpus spongiosum. Microscopically, there is distinct squamous differentiation, papillomatosis, hyper- and orthokeratosis, acanthosis, and a broadly based interface between the tumour and stroma. Koilocytosis is not present. A spectrum of combined tumours exist with focal or significant verrucous features that must be distinguished from pure verrucous carcinomas. These mixed or hybrid verrucous carcinomas are reported to develop metastases in approximately 25% of cases. Associated lesions include: squamous (verrucoid) hyperplasia, differentiated penile intraepithelial neoplasia (PeIN), and lichen sclerosus. No inguinal nodal metastases have been associated with pure verrucous carcinomas and the mortality rate is 0%.

(c) Warty (condylomatous) carcinoma

Warty (condylomatous) carcinomas are slow-growing, low-grade, HPV-related verruciform tumours representing 7%-10% of penile carcinomas. HPV DNA has been detected in 22%-100% of these tumours. Macroscopically, they are cauliflower-like, exophytic, white-to grey tumours. The cut surface shows a papillomatous growth, usually penetrating into the corpora cavernosa or corpus spongiosum. The interface between the tumour and stroma ranges from broadly based to jagged and irregular. Microscopically, the papillae are condylomatous, with a prominent central fibrovascular core and koilocytic changes, not restricted to the surface, but also present in deep invasive portions of the tumour. Hyper- and parakeratosis, cellular pleomorphism, and clear cell features can be observed. A morphologic spectrum of condylomatous tumours exists that share koilocytic changes ranging from clearly benign (usual condylomas) to frankly malignant (invasive warty carcinoma), passing through atypical condylomas and noninvasive warty carcinomas. The biological behavior of warty carcinomas is intermediate between that of verrucous/papillary and PeScc (NOS). Deeply invasive, high-grade warty carcinomas can be associated with inguinal nodal metastases. The differential diagnosis includes verrucous and papillary carcinomas and giant condylomas. Warty carcinomas lack the extreme differentiation of verrucous carcinomas and show jagged and irregular deep borders. Papillary carcinomas lack koilocytosis, and the papillae are more complex, with irregular fibrovascular cores and jagged tumour base. The associated precursor lesions of warty carcinomas are warty/basaloid PeIN. Local recurrences are observed in 10% of cases, and the inguinal metastatic rate is 17%-18%. The cancer-specific mortality rate is 0%-9%.

(d) Papillary carcinoma

Papillary carcinoma is another verruciform tumour and accounts for 5%-15% of penile carcinomas. The HPV detection rate is low. Macroscopically, they are exophytic, large, and irregular. The cut surface shows an invasive papillary neoplasm with an irregular tumour front. Microscopically, the appearance is that of a low-grade papillary squamous neoplasm. There is hyperkeratosis and papillomatosis. The papillae are variable and complex, short or long, with or without a fibrovascular core. The tips are straight, undulated, spiky, or blunt. The tumour front is irregular and infiltrative. Koilocytic changes are absent. The differentiating features from verrucous and warty carcinomas are based on the heterogeneity of the papillae, the lack of koilocytosis, and the jagged irregular interface between the tumour and stroma. The latter feature is crucial to distinguish papillary from verrucous carcinoma. Differentiated PeIN and lichen sclerosus are frequently associated with this PeScc variant. The recurrence rate is low (12%), and inguinal nodal metastases are observed in 12% of all patients. The mortality rate is low (0%-6%).

(e) Basaloid carcinoma

Basaloid carcinoma is an aggressive HPV-related tumour, representing 4%-10% of all penile cancers. About 70%-80% of cases have evidence of HPV infection. Macroscopically, an ulcerated nonexophytic irregular mass is present. The cut surface reveals a tan, solid tumour, deeply invasive into the corpus spongiosum or cavernosum. Basaloid carcinoma limited to the lamina propria is exceedingly rare. Histologically, solid nests of small uniform basaloid cells are found, usually with central necrosis (comedo necrosis) or central abrupt keratinization. Nucleoli are inconspicuous, and apoptosis and mitoses abundant. Warty/basaloid PeIN is often

found in the epithelium adjacent to the invasive cancer. Local recurrences have been observed in up to one third of case and nodal metastasis in 50%-100% of cases. Up to one third of patients will die of systemic metastases, and 10-year survival rate is 76%.

(f) Sarcomatoid carcinoma

Sarcomatoid carcinoma accounts for 1%-3% of penile cancers. It is an aggressive neoplasm predominantly composed of spindle cells. HPV has been found in a few cases. Macroscopically, they appear as bulky, 5-10 cm, ulcerated or rounded polypoid masses. The cut surface almost invariably shows invasion deep into the corpus cavernosum. Microscopically, variable proportions of squamous cell and spindle cell carcinoma are present. The sarcomatoid component might mimic fibrous histiocytoma, leiomyosarcoma, fibrosarcoma, myxosarcoma, or angiosarcoma. Heterologous bone and cartilaginous formation might be focally observed. The differential diagnosis includes sarcoma or malignant melanoma. Inguinal nodal metastasis are seen in 75%-89% of cases, the mortality rate is high (40%-75%), local and systemic recurrences develop in up to two thirds of patients, and most die within 1 year of diagnosis.

(g) Carcinoma cuniculatum

Carcinoma cuniculatum is an extremely unusual verruciform PeScc variant characterized by a burrowing growth pattern. No evidence of HPV infection has been found. Macroscopically, the tumours are white-to-grey and affect the glans, extending to the coronal sulcus and foreskin. Characteristically, on the cut surface, deep invaginations form irregular, narrow, and elongated neoplastic sinus tracts that connect the tumour surface to deep anatomic structures. Microscopically, it resembles

verrucous carcinoma, with a bulbous front of invasion. However, irregular foci of invasive PeScc (NOS) can be present. It should be distinguished from classic verrucous carcinoma, which rarely invades beyond the lamina propria. Despite deep penetration, none of the reported cases have shown groin metastases or systemic dissemination.

(h) Pseudohyperplastic carcinoma

Pseudohyperplastic carcinoma is a clinicopathologic entity represented by low grade, PeScc (NOS) preferentially affecting the foreskin of older patients (eighth decade) in association with lichen sclerosus. The tumour is not related to HPV infection. There is extreme differentiation and, in small biopsies, it can mimic pseudoepitheliomatous hyperplasia. It is often multicentric, and the second or third independent tumour might be verrucous. Macroscopically, they are flat or slightly elevated lesions measuring about 2 cm. Microscopically, keratinizing irregular nests of squamous cells are apparent, with minimal atypia, surrounded by reactive stroma that seems detached from the underlying epithelium and usually invades up to the preputial dartos. The prognosis is excellent, and none of the reported cases showed lymphatic or systemic dissemination.

(i) Adenosquamous carcinoma

Adenosquamous carcinoma is a rare tumour composed of squamous cells intermingled with areas of glandular differentiation. It is thought to arise from the epithelial surface of the glans. Macroscopically, a firm, granular, large neoplasm deeply invading the penile corpora is present. Microscopically, a mixed squamous cell mucin-producing adenocarcinoma pattern is seen. The squamous component

predominates. Glands stain with carcinoembryonic antigen. Adenosquamous carcinoma should be distinguished from mucoepidermoid, adenobasaloid, and pseudoglandular PeScc and from adenocarcinoma arising in the Littré glands. Local recurrences occur in upto 25% of cases. Inguinal node metastases appear in 50% of patients, however, the mortality rate is low (0%-14%).

(j) Acantholytic carcinoma

Acantholytic (adenoid, pseudoglandular) carcinoma is an unusual variant of PeScc characterized by prominent acantholysis and formation of pseudoglandular spaces. Macroscopically, the tumours are large, irregular masses involving multiple penile anatomic compartments and deeply invading into the corpora. Microscopically, the pseudoglandular spaces contain keratin, acantholytic cells, and necrotic debris. Carcinoembryonic antigen and mucin stains are negative. Compared PeScc (NOS), pseudoglandular variants show higher grade foci, invade deeper anatomic structures, and are associated with a greater incidence of regional metastases and mortality.

3.2.3.3. Grading

The "classical" grading system for PeScc described by Broder is based on the degree of cellular anaplasia present (Broders AC. 1921). Well differentiated (G1) squamous carcinoma retains the capability of keratinization with the production of typical keratin pearls. The characteristic intracellular bridges are clearly seen and the degree of anaplasia, as well as the number of mitotic figures are low. Moderately differentiated (G2) squamous carcinoma does not show keratin pearls but only single cell keratinization and a higher number of mitoses with anaplastic cells. Abundant mitoses, poor complete cell differentiation and the lack of keratinization are the

morphological hallmarks of poorly differentiated (G3) squamous carcinomas. Predominantly, a heterogeneous tumour is identified in 53% of cases (a combination of grade 2 and 3). Any proportion of grade 3 is associated with a significant risk of nodal metastasis, suggesting that any focus of grade 3 should be sufficient to grade the neoplasm as high grade.

3.2.3.4. TNM Staging

Historically, several staging systems have been used for PeScc. The Jackson system was introduced in 1966, and the TNM classification was introduced in 1968 and revised in 1978, 1987, 2004 and 2008 (Takayasu and Otaguro 1967; Wehnert, Lohse, and Gunther 1970; Lichtenauer et al. 1972; Chisholm et al. 1994; Leijte et al. 2008). The pT stage is based on the vertical invasion of the different anatomical structures (subepithelial connective tissue, corpus spongiosum or cavernosum, urethra, prostate, adjacent structures). Shortcomings have been observed in the various classifications, and a new clinical staging system was proposed in 1990, with better discrimination of survival rates according to the different stages. Locoregional disease can be subclassified by anatomical level with superficial echelon inguinal lymph nodes draining to deeper inguinal nodes before connecting to the pelvic lymphatics. Poorer survival outcomes are associated with: multiple, bilateral disease; deeper nodal involvement and extranodal extension. Disseminated metastases may be seen in 1-10% of patients at presentation, commonly involving lung, liver, bone and brain.

3.2.3.5. Measurements

Macroscopic measurements of the tumour in three dimensions were recorded where possible. The location of the primary tumour was assessed and the extent of tumour

involvement evaluated with respect to unifocality or multifocality when several sites were affected. All microscopic measurements were calculated using a Vernier's scale and specific distances recorded (Figure 3.1) included:

i. Depth of invasion (mm) – measured from adjacent epithelial surface to the deepest infiltrating edge or nest of tumour cells.

ii. Resection margins (mm) – measured for the deep resection and skin excision limits. Margins were inked prior to specimen processing for clarity.

3.2.3.6. Immunohistochemistry

Tissue sections prepared as described in section 3.2.1 with endothelial markers CD31 and CD34, were carefully scrutinised for evidence of vascular invasion. This was defined by tumour thrombus within a vascular space and differentiated from retraction artefact.

3.3. Results

3.3.1. Clinicopathological information

Clinical data for 141 patients diagnosed with carcinoma in situ (Cis) or invasive squamous carcinoma of the penis (PeScc) were entered into the study database. Patients with carcinoma in situ (n=12) or incomplete follow up data (n=11) were excluded from all analyses. Table 3.1 summarises the clinicopathological characteristics of the patients. The mean age of all patients at the time of diagnosis was 60.1 years (range 27 to 87 years).

The primary tumour was treated by local excision or circumcision in 27 cases, partial or total glans excision in 50 cases and partial or total penectomy in 49 cases. 3 cases were treated by total urethrectomy. Regional lymph node dissection (superficial +/- deep inguinal lymph nodes) was performed in 71 patients. Additionally, 25 patients were under active surveillance for more than two years and 20 patients for less than 2 years. Furthermore, it was not possible to determine the lymph node status in a further 2 patients. PeScc was staged according to 2004 TMN staging criteria and were classified as pT1 in 47% (n=60), pT2 in 43% (n=55), pT3 in 9% (n=12) and pT4 in 1% (n=2). There were no superficial pTa lesions recorded in this series. A total of 37 patients (29%) were diagnosed with positive inguinal lymph node involvement and 59 patients (46%) classified as inguinal node negative. 33 patients (25%) remained unstaged (Nx) with respect to nodal status.

At the time of diagnosis two patients had clinical or radiological evidence of distant metastases. Histological tumour types were grouped as PeScc: NOS (n=87), papillary (n=18), basaloid (n=7), warty/verrucous (n=6) and mixed/hybrid (n=11). Tumour grade was recorded as well differentiated (G1) in 26 patients, moderately differentiated (G2) in 54 patients and poorly differentiated (G3) in 49 patients. Overall, vascular invasion was identified in 28 cases (22%), BXO seen within 15 specimens (12%) and 28 patients (22%) were classified with multifocal tumour patterns. 60 tumours (57%) were classified as greater than 2cm in size and overall distribution of the depth of invasion was less than 5mm (n=58), 5-10mm (n=27), 11-20mm (n=13) and greater than 20mm (n=15).

Table 3.1

3.3.2. DNA content and correlation to conventional tumour pathology

DNA distribution histograms from 112 patients were classified as diploid in 41 cases (34%) and aneuploid in 71 cases (66%) based on the classification system outlined in section 2.7. The correlation of DNA content with available clinical data and tumour pathology is summarised in Table 3.2. Aneuploidy was significantly associated with increasing tumour anaplasia ($p<0.0001$) demonstrating a strong link between the loss of differentiation programmes and genomic instability. Interestingly, it was not possible to establish an association between aneuploidy and other traditional pathological factors. From these data, it is observed that DNA content can easily be measured in PeScc and may add independent information if included in diagnostic and prognostic algorithms.

3.3.3. Correlation between histopathological factors, DNA content and lymph node involvement

Accordingly, I sought to assess the univariate associations between tumour pathology variables (conventional histopathology and DNA content) and lymph node status (positive and negative) using a logistic regression model. Information for 96 men was entered into this analysis and univariate associations were calculated. Predictors which had statistically significant associations (at the 5% level) were investigated further using multivariable models (Table 3.3). Significant predictors of lymph node involvement ($P<0.05$) in this series include: grade, stage, aneuploidy and vascular invasion. Notably, a strong association between aneuploid tumours and distant metastases (metastases positive; $p=0.04$) was also observed. Multivariate analysis with backward elimination resulted in ploidy status and vascular invasion being dropped from the final model. Tumour grade and stage prove to be the strongest

Table 3.2

independent predictors for lymph node positivity (Table 3.4). The ROC area for this model was 0.78 (95% CI: 0.69 to 0.87). Given that the regression coefficients are simply the log of the odds ratios, it is possible to calculate the log-odds for an individual patient; thereby estimating their risk for lymph node metastases. Using the formula:

$$log[p/(1-p)] = -3.410 + 1.569xGrade(2) + 2.628xGrade(3)$$
$$+ 1.933xStage(2) + 0.842xStage(3)$$

where p is the probability of lymph node disease. For example, a patient with PeScc (G3pT2) has log-odds -3.410 + 2.628 + 1.933 = 1.151. This can then be transformed to the risk of node involvement in this patient of 76%.

3.4. Discussion

A number of potential clinical and pathological prognostic variables have been proposed in PeScc. Integrating these data generates a novel staging system for predicting locoregional involvement in patients with PeScc. Published nomograms incorporate the following information: clinical inguinal lymph node stage, pathologic tumour thickness, growth pattern, histologic grade, lymphatic and/or venous embolization, corpora cavernosa infiltration, corpus spongiosum, and/or urethral infiltration (Solsona et al. 2004; Ficarra et al. 2006; Kattan et al. 2006b; Kattan et al. 2006a). I have shown through the work conducted in this chapter that many of these

Table 3.3

Table 3.4

variables are reproducible in this series. Additionally I have identified a novel discrete, binary variable that can be easily determined on initial pathological assessment. Ploidy status (diploid or aneuploid) determined by image cytometry offers novel, alternative information when staging penile cancer patients at an early stage in there overall treatment.

Tissue DNA content can be assessed by either flow (FCM) or image (ICM) cytometry which detect gross genomic aberrations. A number of advantages have been proposed that support the use of ICM over FCM (Baretton et al. 1994a); however the two methods have shown high levels of concordance in various studies (Kachel et al. 1979; Haroske et al. 2001; Bol et al. 2003; Friedrich et al. 2004; Wohlrab et al. 2005; Tarnok, Valet, and Emmrich 2006; Huang et al. 2008; Kilpatrick et al. 1994; Borgiani et al. 1994). It is understood that ICM offers increased sensitivity to detect aneuploidy, the presence of an internal reference standard, and the ability to generate quality histograms uncontaminated by debris and clumps of nuclei. FCM is limited by the potential for cell loss leading to erroneous aneuploidy measurements, does not permit morphological confirmation of measured objects and multinucleated histiocytes contribute both to high coefficients of variation (CV) and to the so-called "ripple effect". Computed image cytometry offers an automated platform for analysis and is particularly useful for clarifying difficult areas in flow histograms - specifically, high coefficients of variation, high G2M phase, as well as possible near diploid aneuploidy and hypodiploidy. Finally, ICM has been successfully utilised in determining DNA content in a number of different solid tumours and linked to pathological and survival data (Theissig et al. 1991; Bottger et al. 1991; Bottger et al. 1992; Steinbach et al. 1993; Baretton et al. 1994b; Baretton et al. 1995; Sampedro et

al. 1996; Schimming et al. 1998; Bottger et al. 1999; Riesener et al. 1999; Grote et al. 2001; Raatz, Bocking, and Hauptmann 2004; Melegh et al. 2005; Lexander et al. 2006; Cai et al. 2006; Yildirim-Assaf et al. 2007). The results presented in this chapter support the hypothesis that aneuploid tumours behave in a more aggressive fashion and are associated with locoregional and disseminated metastases in these patients.

Currently, the impact of tumour ploidy status as a prognostic factor in PeScc is limited to a few small series and isolated case reports using FCM. Masih et al described a diploid population of tumour cells in all verrucous carcinomas studied in their series. These tumours are known to be well differentiated and behave in an indolent biological fashion; supporting the finding of euploidy in this study. Ornellas et al performed FCM in 90 cases demonstrating that aneuploidy rates increased with tumour grade: well differentiated (5.5%), moderately differentiated (28.8%) and poorly differentiated (66.6%). Unfortunately, there were only 3 poorly differentiated tumours in this study, preventing formal analysis of aneuploidy as a prognostic factor. Nonetheless, there was a tendency towards high DNA content correlating with increased metastatic risk. Hall et al failed to demonstrate a prognostic predictive value in 46 men for ploidy determined by FCM above standard pathological parameters used at that time (Hall et al. 1998). No formal survival analysis was undertaken in this study and limitations with FCM methodology and histopathological assessment may account for differences seen against the results presented in this chapter. Winkler et al analysed 30 urethral squamous carcinomas by FCM and showed strong associations between aneuploidy and 5yr/10yr survival rates.

I have illustrated that aneuploidy is significantly associated with increasing tumour anaplasia in 112 cases of PeScc; demonstrating a strong link between the loss of differentiation programmes and genomic instability. In chapters five and six, I expand on these findings to link aneuploid states with cell cycle dysregulation and explore the utility of image cytometry as a prognostic tool in predicting survival outcomes in PeScc.

Current EAU guidelines stratify the risk of lymph node involvement by the grade and stage of the tumour in conjunction with clinical nodal status. The results presented here, demonstrate that grade and stage remain powerful, independent predictive variables for lymph node disease. Unfortunately, given the retrospective nature of the study, it was not possible to incorporate some important variables such as clinical stage into this model. Prospective analysis including these variables with the additional information offered by image cytometry may provide improved power to the current integrated staging systems available. This will allow earlier risk stratification with improved counselling of patients and better allocation to surveillance or radical treatment programmes.

3.5. Conclusion

Image cytometry offers a novel and reproducible technique for determining the nuclear DNA content of tumour cells in patients with penile cancer. I have demonstrated that tumour ploidy status provides powerful prognostic information alongside conventional histopathology factors. This novel approach may permit early stratification of patients for aggressive surgical and chemotherapeutic treatments.

Further large scale studies are warranted to support the careful incorporation of DNA aneuploidy into diagnostic and treatment guidelines for this unusual malignancy.

CHAPTER FOUR

DEFINING NORMAL AND ABBERANT REGULATION OF THE DNA REPLICATION LICENSING SYSTEM IN PENILE SQUAMOUS CELL CARCINOMA

CHAPTER FOUR

DEFINING NORMAL AND ABBERANT REGULATION OF THE DNA REPLICATION LICENSING SYSTEM IN PENILE SQUAMOUS CELL CARCINOMA

4.1. Introduction

The DNA replication licensing pathway has emerged as a powerful downstream mechanism for controlling the proliferative state of cells and ensures that DNA is replicated once and only once per cell cycle; thus maintaining genomic stability (Blow and Hodgson 2002a; Blow and Dutta 2005; Machida, Hamlin, and Dutta 2005; Jackson, Laskey, and Coleman 2013). Complex, multifunctional pathways are important in the coordinated growth of tissue systems in multicellular organisms. However, it has been shown that only a small proportion of cells are actively engaged in proliferative mechanisms within multicellular tissues. As described in detail in section 1.2, this observation has led to the identification of distinct cellular phenotypes relating to their respective withdrawal from the cell division cycle. Hence, cells may be either permanently or temporarily withdrawn from the cell cycle as a result of: terminal differentiation, quiescence or senescence (Hall and Watt 1989; Williams and Stoeber 2007). G0 represents a state when cells have reversibly withdrawn from the cell division cycle in response to high cell density or mitogen deprivation (Zetterberg 1985). A minority of cells are actively cycling (proliferating) and these are located mainly in the stem-transit amplifying compartments of self-renewing tissues, such as epithelia and bone marrow (Potten and Loeffler 1990).

In section 1.4, I described the utility of RLFs as sensitive detectors for distinguishing cell cycle phase-specific cell populations. During late mitosis and early G1 phase, the replication licensing factors: ORC, Cdc6, Cdt1, and Mcm2-7 assemble into pre-replicative complexes, which render replication origins "licensed" for DNA synthesis. During S phase, Cdc7 kinase and cyclin-dependent kinases induce a conformational change in the pre-replicative complex, resulting in recruitment of additional initiator proteins that collectively promote DNA unwinding and recruitment of DNA polymerases. During S-G2-M phases, the presence of the licensing repressor protein geminin prevents inappropriate re-initiation events at origins that have already been activated (Sclafani and Holzen 2007; Williams and Stoeber 2007; Blow and Gillespie 2008).

Mcm2-7 (MCM) proteins are expressed throughout the cell cycle (G1-S-G2-M) but are tightly downregulated during exit into out-of-cycle quiescent (G0), differentiated, or senescent states (Stoeber et al. 1998; Stoeber et al. 2001; Blow and Hodgson 2002b; Eward et al. 2004; Kingsbury et al. 2005; Barkley et al. 2007; Williams and Stoeber 2007) [Figure 4.1]. Unlicensed replication origins and absence of CDK activity characterize these out-of-cycle states and therefore allow such cells to be clearly distinguished from cycling cells in complex and dynamic heterogeneous cell populations (Stoeber et al. 2001). Normal somatic cells demonstrate a key 'proliferation-differentiation' switch which is responsible for the withdrawal of cells from the mitotic cell cycle. These mechanisms are essential for the fundamental and normal function of different cells types within a tissue system and contribute to the

Figure 4.1

loss of proliferative capacity as cells transit in to out of cycle states. Thus, the MCM proteins represent novel biomarkers of growth and have been confirmed as powerful markers for cancer detection and prognostication in a wide range of tumour types (Gonzalez et al. 2005; Williams and Stoeber 2007; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013). Moreover, expression profiling of MCMs together with Ki67 (a standard proliferation marker) and geminin (biomarker of S-G2-M progression) allows cells in out-of-cycle states to be distinguished from those residing in cycle and can assign cells to G1 and S-G2-M phases (Kulkarni et al. 2007; Loddo et al. 2009). Mcm2-7 protein expression also identifies non-cycling cells with proliferative potential (Figure 4.2). For example, in luminal breast epithelium of pre-menopausal women, a large proportion of cells reside in a primed 'replication licensed' but non-proliferating state, characterised by expression of Mcm2 but absence of Ki67 and geminin (Williams et al. 1998; Stoeber et al. 1999; Stoeber et al. 2001; Going et al. 2002; Shetty et al. 2005; Williams and Stoeber 2007). This phenomenon exemplifies the unique proliferative characteristics of these cells compared to tissues in the post-menopausal cohort. It is believed that this represents an evolutionary preserved mechanism allowing rapid cellular expansion secondary to hormonal fluctuations observed in pregnancy and lactation (Stoeber et al. 2001; Blow and Hodgson 2002b; Williams and Stoeber 2007; Loddo et al. 2009; Williams and Stoeber 2012)

The ability of cells to re-enter the cell cycle after a period of non-proliferative activity, provides a crucial mechanism through which multicellular organisms can respond to mitogenic stimuli (i.e. tissue damage leading to healing and repair). The

Figure 4.2

relative ease in which cells are capable of making this transition has led to the classification of human tissues into three main groups. (1) Self-renewing tissues (e.g. skin, gastrointestinal mucosa and haematopoietic system) demonstrate rapid and continuous cell turnover. (2) Conditional renewal tissues (e.g. liver and thyroid) respond rapidly, as outlined above, to mitogenic stimuli with variable periods of stable inactivity. Finally, (3) Permanent tissues (e.g. nerve and skeletal muscle) are predominately composed of terminally differentiated cells and therefore lack the capacity for self-renewal.

An *in vivo,* functional model demonstrating these processes is observed when the expression of MCM proteins are analysed in the human colonic crypt. As a self-renewing tissue, normal colon shows the capacity for continuous cell turnover, through distinct proliferative compartments; thus maintaining the integrity and function of the terminally differentiated epithelial cells located at the luminal surface (Potten and Loeffler 1990). The highest level of MCM expression is observed in the transit amplifying population of cells. The level of MCM expression subsequently declines in the middle third of the crypt, becoming undetectable in surface terminally differentiated cells (Stoeber et al. 2001).

Thus, combinatorial analysis of these important effectors of cellular growth and proliferation can provide an important insight into the cellular kinetics of different tissue systems. The Mcm2/Ki67 ratio defines the proportion of cells that are licensed to proliferate. Consequently, the higher the Mcm2/Ki67 ratio, the greater the proportion of cells that reside in a licensed non-cycling state (Dudderidge et al. 2005; Shetty et al. 2005; Kulkarni et al. 2007; Williams and Stoeber 2007; Loddo et al.

2009). Because Ki67 is present throughout the cell cycle in proliferating cells, the geminin/Ki67 ratio may be used as an indicator of the relative length of G1 phase and the rate of cell cycle progression (Dudderidge et al. 2005; Shetty et al. 2005; Kulkarni et al. 2007; Williams and Stoeber 2007; Loddo et al. 2009). Proliferating cells with a short G1 phase will approximate to a geminin/Ki67 ratio of ~1; whereas cells with a prolonged G1 phase will approximate to a ratio closer to 0 (Dudderidge et al. 2005; Obermann et al. 2005; Shetty et al. 2005; Dudderidge et al. 2007; Kulkarni et al. 2007; Loddo et al. 2009)). Similarly, the Ki67-geminin labeling index (LI) can be used to identify the numbers of cells transiting G1 phase (Wharton et al. 2004; Dudderidge et al. 2005; Dudderidge et al. 2007). Importantly, studies show that this method of multiparameter analysis of RLFs translates into prognostic information with regards to patient survival in several tumour types (Dudderidge et al. 2005; Obermann et al. 2005; Shetty et al. 2005; Dudderidge et al. 2007; Kulkarni et al. 2007; Loddo et al. 2009).

Tumours acquire a growth advantage over normal tissues through a variety of mechanisms, including acquisition of aneuploidy and dysregulation of the mechanisms that control cellular proliferation. Complex signaling pathways interlinked with redundant growth-regulatory mechanisms contribute to the diverse and heterogeneous effects of oncogenic mutations observed in diverse tumour types. Dysregulation of replication licensing in early tumourigenesis may arise as a consequence of oncogene-induced cell proliferation, which can cause under replication or over replication of chromosomal DNA and therefore contribute to the development of aneuploidy commonly seen during multistep tumour progression to an aggressive cancer phenotype (Vaziri et al. 2003; Blow and Dutta 2005; Zhu and Dutta

2006; Lin and Dutta 2007). MCM proteins represent novel biomarkers of growth and have been confirmed as powerful markers for cancer detection and prognostication in a wide range of tumour types (Williams et al. 1998; Freeman et al. 1999; Stoeber et al. 1999; Going et al. 2002; Stoeber et al. 2002; Gonzalez et al. 2003; Williams et al. 2004; Dudderidge et al. 2005; Obermann et al. 2005; Shetty et al. 2005; Dudderidge et al. 2007; Kulkarni et al. 2007; Scarpini et al. 2008; Loddo et al. 2009; Dudderidge et al. 2010; Kelly et al. 2012; Saeb-Parsy et al. 2012).

Through greater understanding of the RLF pathway in malignancy, it has been shown that aberrant DNA licensing is a common finding in many solid tumours leading to an increased proliferative capacity in cells that would otherwise demonstrate down-regulation of these key proteins. This event is thought to occur at an early point in tumourigenesis and provides insight into the mechanisms that may be involved in the malignant transformation of epithelial cells (Williams and Stoeber 2007; Blow and Gillespie 2008; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013). Crucial understanding of these mechanisms remains to be explained in penile epithelium and the subsequent dysregualtion that may be observed in penile carcinoma evaluated. In light of these observations, I have sought to investigate the dysregulation of RLFs, in order to elucidate their role in penile epithelial carcinogenesis. Furthermore, I have used multiparameter analysis of Mcm2, Ki67 and geminin in PeScc, to gain insight into the *in vivo* cell cycle kinetics of this heterogeneous and complex tumour type.

4.2. Materials & Methods

For a detailed methodological summary refer to the following subsections in chapter

two: Antibodies (2.3.1), Immunoblotting (2.3.2), Immunohistochemistry (2.3.3), Protein expression profile analysis (2.3.4), Cell culture and synchronisation (2.5.1), Preparation of total cell extracts (2.5.2), Statistical methods (2.6).

4.3. Results

4.3.1. Cell cycle phase specific expression of replication licensing factors

Extensive characterisation of the RLF antibodies (section 2.3.1) used throughout these experiments has been undertaken and published widely. Detailed data recorded previously has been reconciled using both *in vitro* and *in vivo* systems with regard to protein monospecificity, immunolocalisation in conjunction with peptide blocking experiments. Initially, I sought to demonstrate the regulation of the DNA replication licensing system in different *in vitro* systems; thereby consolidating the work detailed above. Prior to investigation of these cell cycle regulators in synchronous cell extracts, the monospecificity of these antibodies against Mcm2, Cdt1, geminin was confirmed in asynchronous HeLa cells by detection of a single protein with a molecular mass consistent with the reported electrophoretic mobility of the corresponding human antigen (Figure 4.3). Similar data for Mcm2 and geminin has previously been described (Wharton et al. 2004).

Subsequently, in order to further delineate the monospecificity of these antibodies, I performed Western blot analysis on extracts prepared from a synchronous cell populations using HeLa and SKOV-3 cells as described in sections 2.3.2, 2.4.1 & 2.4.2. The aim of this series of experiments was to clarify the expression of RLFs with respect to normal cell cycle phase analysis. Technical support was gratefully received from Dr Sarah Kingsbury and Mr Marco Loddo with regards to acquiring these data.

Synchronized cycling cells were cultured and cell phase specificity determined using bivariate FACS (section 2.4.3). I proceeded to immunoblot protein extracts prepared from these cells with the monospecific antibodies listed in section 2.3.1 (Figure 4.4). I observed that Mcm2 levels did not vary significantly during passage through the cell cycle. These findings recapitulate the data from studies in budding yeast which demonstrate that MCM proteins attach and detach from chromatin but are not degraded. Rather MCM complexes undergo exportation out of the nucleus in preference to overall inhibition (Siddiqui, On, and Diffley 2013).

Geminin expression was restricted to S-G2-M phases of the cell cycle as reported previously (Wharton et al. 2004) . This is in keeping with geminin's inhibitory role on Pre-RC assembly and is governed by its degradation in late mitosis and G1 by the ubiquitin-protein ligase APC. Levels of geminin are kept low in G1 thereby promoting the role of Cdt1 in Pre-RC assembly. I observed that Cdt1 levels began to rise at the end of mitosis, increased during G1 and fell abruptly at the start of S-phase. This highlights the critical interplay between rising geminin levels when the APC is inactivated and Cdt1 which is inhibited by geminin and degraded through targeted ubiquitination by SCF complex (a multi-protein E3 ubiquitin ligase complex) under CDK control (Siddiqui, On, and Diffley 2013). Actin levels were used as an internal control.

Finally, further supportive data is gained from published immunoblots of total cell extracts for cells undergoing terminal differentiation (Barkley et al. 2007). In this HL60 cellular model, a clear down-regulation of Cdc6 is observed as an early event in this process occurring at 12h. Subsequent fall in other RLFs is noted as these cells

progress to a non-cycling population with a terminally differentiated phenotype at 144 hours. Cdc6 expression appears to represent a rate-limiting step in this process. Conversely, cells re-entering the cell cycle from a quiescent state appear to up-regulate the RLFs early in the transition from G0. Ectopic geminin can block re-acquisition of DNA replication competence during re-entry into the cell cycle, indicating that geminin levels must be tightly down-regulated for escape from G0.

In summary, these *in vitro* data recapitulate the published findings regarding the tight cell cycle phase-specific expression of RLFs. All antibodies, shown here, have a high level of monospecificity for their relative human antigen targets. Finally, it is observed that cells with intact "proliferation-differentiation" programmes can tightly regulate RLF expression to control exit to a G0 state. Subsequently, I will explore how RLF expression varies in different tumour groups in PeScc reflecting the loss of these important controls with increasing anaplasia when compared to the normal cell types studied here.

Figure 4.3

Figure 4.4

4.3.2. Regulation of the DNA licensing system in normal and dysplastic penile epithelium

Next, I undertook further experiments using these monospecific antibodies to investigate the dysregulation of DNA replication licensing system in early, multistep progression of tumour development observed in normal and dysplastic penile epithelium. Here, I explore the protein expression profiles for Mcm2 and geminin, against the standard proliferation marker Ki67, in normal penile tissue alongside dysplastic epithelium and invasive PeScc. I sought to determine the spatiotemporal relationship of RLF and Ki67 expression using immunohistochemical staining in representative cases and to quantify the changes detected in normal, dysplastic and neoplastic penile epithelia. Furthermore, I investigated the relationship of dysregulated RLF expression with increasing anaplasia reflecting diminished cellular differentiation.

4.3.2.1. Normal penile epithelium

In normal penile squamous epithelium, RLF and Ki67 expression is restricted to the basal and suprabasal layers consistent with the transamplifying compartment (TAC) seen in other self-renewing systems (Freeman et al. 1999; Stoeber et al. 2001; Williams and Stoeber 2007; Jackson, Laskey, and Coleman 2013) [Figure 4.5]. Cells in the superficial layers demonstrate a fully differentiated phenotype with flattened morphology and stain negative for Mcm2, Ki67 and geminin expression. The level of RLF and Ki67 expression was extremely low (<4%) which is consistent with the previous finding that the loss of proliferative capacity which accompanies differentiation is coupled to down-regulation of the RLFs involved in origin licensing

(Mcm2-7, Cdc6 and Cdt1) (Barkley et al. 2007; Williams and Stoeber 2007; Williams and Stoeber 2012).

4.3.2.2. Dysplastic penile epithelium

In contrast dysplastic lesions demonstrate high RLF and Ki67 expression reflecting discordant differentiation processes and an expanded proliferative compartment (Figure 4.5). It is observed that as tissues advance from mild or moderate dysplasia to full thickness dysplasia (carcinoma in-situ); there is a corresponding increase in the expression of Mcm2. These data mirror the findings observed in pre-malignant conditions of the uterine cervix, colon and oesophagus (Williams et al. 1998; Freeman et al. 1999; Going et al. 2002; Stoeber et al. 2002; Williams et al. 2004; Laskey 2005; Scott et al. 2006; Williams and Stoeber 2007; Williams and Stoeber 2012); thereby underpinning the theoretical benefits of assessing Mcm2 levels in screening tests, currently under review in a number of international clinical trials (Siddiqui et al. 2008; Tambouret, Misdraji, and Wilbur 2008; Wilbur et al. 2009; Depuydt et al. 2011). These principles may be of clinical use in identifying severe dysplastic lesions on male genital epithelium.

4.3.3. Regulation of the DNA licensing system in neoplastic penile epithelium

RLF and Ki67 expression was significantly greater in malignant tissue compared to normal epithelium. Interestingly, I observed that biomarker immunostaining at the advancing tumour edge in low grade tumours (i.e. the proliferative compartment) recapitulates the findings demonstrated in the *in vitro* HL60 differentiation model (Musahl et al. 1998; Barkley et al. 2007). These tumours stained exclusively at the

Figure 4.5

Figure 4.6

peripheral tumour edge in keeping with a biologically active compartment. However, biomarker expression is sharply down-regulated as functional differentiation programmes are initiated towards the centre of these tumours. These cells appear to retain the capacity to differentiate and thus withdraw to a G0 state; reflecting a lower growth potential. In sharp contrast, there is a greater degree of biomarker staining throughout the tumour in high grade cancers; which reflects the loss of normal differentiation mechanisms, thereby retaining a larger proportion of cells in the cell division cycle (Figure 4.6). Furthermore, an increased expression of the S-G2-M phase-specific marker geminin with increasing anaplasia suggests that higher grade tumours yield greater proliferative fractions compared to low grade tumours.

Analysis of the labelling indices (LI) [i.e. percentage of positively staining cells] (appendix B) revealed interesting characteristics with respect to biomarker expression against tumour grade. The distribution of LIs (median, inter-quartile range) for each biomarker is presented (Figure 4.7). Mcm2 protein expression (median LI) was greater than Ki67 expression (median LI), with both biomarkers mapped over a broad range. Mcm2 and Ki67 expression levels were significantly higher than geminin expression in these tumours, reflecting the lower growth fraction identified by geminin, which is exclusively expressed during the S-G2-M phases of the cell cycle. There exists a strong correlation between all biomarkers, highlighted by the high degree of concordance, from low to high scores, for Mcm2 and Ki67 (ρ=0.87), confirming the use of Mcm2 as a proliferation marker.

4.3.4. Dysregulation of RLF expression with increasing anaplasia in penile carcinoma

I reviewed the protein expression profiling in PeScc specimens in order to better understand the changes observed when tissues lose their normal differentiation mechanisms in connection with increasing anaplasia. In well differentiated PeScc (grade 1), I observed that RLF and Ki67 expression is restricted to the peripheral zone of tumour nests. Central zones in these tumours stained negatively for all three biomarkers reflecting their non-cycling, non-proliferating state (Figure 4.6). This replicates the *in vitro* studies using the HL60 model as described earlier. It is likely that these cells are permanently withdrawn from the cell cycle and is coupled to reduced proliferative capacity. This recapitulates the proliferation-differentiation switch seen in normal, self-renewing tissues (Stoeber et al. 2001; Williams and Stoeber 2007; Williams and Stoeber 2012). In contrast, moderate and poorly differentiated PeScc (grades 2 and 3) demonstrate a greater degree of "maturation arrest" which is linked to a higher proportion of tumour cells being engaged in the cell division cycle (i.e. a higher growth fraction). Also, this recapitulates the high proliferation signature observed in full-thickness dysplastic lesions also showing maturation arrest (Figures 4.7 & 4.8). This is indicative of cell cycle engagement and an increase in proliferative capacity.

I observed a significant correlation for increasing LI ($p<0.0001$) with increasing grade for all biomarkers (Figure 4.8; Table 4.1). It was also observed that when compared to other tissue types and specific cancers (Gonzalez et al. 2003; Wharton et al. 2004; Dudderidge et al. 2005; Obermann et al. 2005; Shetty et al. 2005; Mehrotra et al. 2006; Dudderidge et al. 2007; Kulkarni et al. 2007; Loddo et al. 2009; Williams and

Stoeber 2012), the Ki67 expression almost matches Mcm2 expression in moderate and poorly differentiated tumours. This finding demonstrates that with increasing anaplasia, cells are licensed and cycling as seen in other squamous cell carcinomas (Williams et al. 1998; Kodani et al. 2001; Going et al. 2002; Chatrath et al. 2003; Williams et al. 2004; Gonzalez et al. 2005; Scott et al. 2006; Williams and Stoeber 2007).

Figure 4.7

Figure 4.8

Figure 4.9

Table 4.1

4.3.5. Cell cycle kinetics with increasing anaplasia in penile carcinoma

The 'Ki67-geminin' score was associated with an increase in tumour grade ($P < 0.0001$), indicative of an increase in the number of cells transiting G1 phase (Figure 4.9). Thus, the proportion of tumour cells actively cycling increases with increasing grade. There was little evidence, however, of an increase in the geminin/Ki67 ratio with increasing grade. This ratio is an indicator of the relative length of G1 phase and the results suggest that increased recruitment of cells into the cell division cycle was not linked to accelerated cell cycle progression as seen in other tumour types (e.g., epithelial ovarian cancer) (Kulkarni et al. 2007). There was evidence of a trend for decreasing Mcm2/Ki67 ratio with increasing grade ($P = 0.09$); reflecting a shift in the proportion of non-proliferating cells that are licensed for DNA replication in well-differentiated tumours to a population of actively cycling cells in poorly differentiated tumours (Dudderidge et al. 2005; Kulkarni et al. 2007; Loddo et al. 2009).

4.4 Discussion

Detection of Mcm 2-7 can help describe the proliferative state and growth fraction in dynamic cell populations (Stoeber et al. 2001; Williams and Stoeber 2007; Williams and Stoeber 2012). Repression of the DNA replication licensing machinery is a powerful mechanism through which cells can lower their proliferative capacity on exiting from the cell cycle in to a differentiated state. Here, I have clearly demonstrated that DNA replication licensing in normal penile epithelium conforms to the principles seen in other self-renewing tissues (Stoeber et al. 2001; Williams and Stoeber 2007; Williams and Stoeber 2012). The putative stem cell compartment in squamous epithelium may reside in the basal compartment from which proliferating cell colonies propagate according to Potten's model (Potten and Loeffler 1990)

outlined in chapetr 1. As, these cells enter the transamplification compartment (TAC) in penile squamous epithelium, they stain positively for Mcm2, Ki67 and geminin indicative of a fully proliferating cell. This effect is clearly observed in other squamous epithelium (e.g. oesophagus) and the colonic crypt (Stoeber et al. 2001). Early differentiating cellular populations demonstrate a subset of cells expressing Mcm2 alone, with Ki67 and geminin rapidly downregulated during the proliferation-differentiation "molecular" switch. The inverse relationship between RLF expression and differentiation status recapitulates the findings in the *in vitro* HL60 monocyte/macrophage differentiation model system (Barkley et al. 2007) and has been noted in several other malignancies (Williams et al. 1998; Freeman et al. 1999; Meng et al. 2001; Going et al. 2002; Chatrath et al. 2003; Gonzalez et al. 2003; Williams et al. 2004; Dudderidge et al. 2005; Obermann et al. 2005; Shetty et al. 2005; Dudderidge et al. 2007; Kulkarni et al. 2007; Ayaru et al. 2008). Terminally differentiated cells reflect a complete withdrawal from the cell cycle and repression of origin licensing (Stoeber et al. 2001; Blow and Hodgson 2002b; Eward et al. 2004; Williams and Stoeber 2007). I have shown that cell systems in normal penile epithelium and PeScc, in which differentiation programmes remain functional, that transition to terminally differentiated "out of cycle" states is tightly coupled to early downregulation of RLFs which recapitulates what is seen in the HL60 systems (Musahl et al. 1998; Barkley et al. 2007). Ultimately, this reflects that the majority of the critical components of the RLF pathway are under E2F regulation (Fang and Han 2006; Blow and Gillespie 2008; Tudzarova et al. 2010). Hence, the RLF pathway can be regulated at two levels: (1) at the transcription level, by E2F and growth-arresting signals, and (2) posttranscriptionally, by CDK phosphorylation, a step that is required

for eventual proteasome-mediated degradation of critical RLFs (Sclafani and Holzen 2007; Blow and Gillespie 2008; Williams and Stoeber 2012).

In contrast, higher grade cancers are characterised by uncontrolled growth with increased proliferation and abrogation of cell cycle checkpoints (Nurse 2002). In PeScc, this biological phenomenon is exemplified by high RLF and Ki67 expression indicative of cells failing to withdraw from the cell division cycle in keeping with a failure of the proliferation-differentiation switch and the onset of maturation arrest (i.e. a block to the differentiation programme). Furthermore, dysregulation of origin licensing and proliferative pathways with increasing anaplasia reflects the loss of normal somatic differentiation programmes and up-regulation of proliferation genes, notably Mcm 2-7 expression (Van't Veer and Bernards 2008). Crucially, cancers represent a heterogeneous population of cycling and non-cycling cells. As a tumour becomes more poorly differentiated there is a switch to a predominantly cycling and growing phenotype. In this study, it is observed that only a small fraction of cells may be cycling in well-differentiated, low-grade tumours; whilst the majority of cells reside in the fully differentiated 'out-of-cycle' state. Hence, these G1/S regulators of the cell cycle are critically involved in allowing cells to replicate and can sensitively predict cells residing "in cycle".

The HL60 monocyte/macrophage differentiation model system (Barkley et al. 2007) illustrates that loss of proliferative capacity and cell cycle withdrawal following engagement of the somatic differentiation program is tightly coupled to downregulation of core constituents of the DNA replication licensing machinery including the Mcm2-7 proteins. This coupling between loss of proliferative capacity,

cell cycle withdrawal, downregulation of the Mcm2-7 helicase complex, and differentiation has been observed in anal, bladder, cervical, colonic, esophageal, oral, pancreatic, and prostatic epithelia (Williams et al. 1998; Kodani et al. 2001; Williams et al. 2004; Scott et al. 2006; Ayaru et al. 2008; Scarpini et al. 2008; Dudderidge et al. 2010). In normal stratified squamous penile epithelium, I observed downregulation of RLFs as cells exit the cell cycle and engage normal somatic differentiation programs. This seems to be a ubiquitous mechanism for lowering the proliferative capacity of cells in stem-transit–differentiating self-renewing tissue systems (Stoeber et al. 2001). In contrast, the block to normal differentiation programmes (i.e. arrested differentiation) that characterizes dysplastic (pre-invasive) lesions is associated with persistent expression of MCM proteins even in surface epithelial layers which is indicative of cells failing to withdraw from the cell cycle. As previously observed for dysplastic lesions of the cervix, esophagus, bladder, oral, and anal mucosa (Williams et al. 1998; Stoeber et al. 2002; Williams et al. 2004; Scott et al. 2006; Ayaru et al. 2008; Scarpini et al. 2008; Dudderidge et al. 2010; Kelly et al. 2012), high-level MCM expression was detected in penile dysplasia. Interpreting these results, it would be possible to surface sample new or recurrent penile lesions (e.g. brush cytology) followed by immunoexpression analysis for MCM proteins in order to provide a rapid method for distinguishing benign hyperplastic lesions from dysplasia. This approach has already been exploited in screening for cervical cancer and detection of oesophageal, lung, bladder, anal, and oral dysplasia (Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013).

Cell cycle phase analysis allows an accurate assessment of individual tumour kinetics (Figure 4.10) which imparts, not only important prognostic information, but also

predictive potential for response to phase specific drugs. The disappointing intent-to-treat analyses of large, conventionally designed trials, such as TACT and tAnGo, suggests that further improvements in adjuvant treatment will require individualized therapeutic decisions (Wardley et al. 2008; Ellis et al. 2009). Cell cycle phase analysis of breast and ovarian cancers has shown that it is tumours displaying the accelerated cell cycle phenotype that are most likely to show a clinically relevant response to S- or M-phase-directed agents (Kulkarni et al. 2007; Williams and Stoeber 2007; Kulkarni et al. 2009; Loddo et al. 2009; Rodriguez-Acebes et al. 2010; Williams and Stoeber 2012). As non-proliferating cells are radiation-resistant, whereas cycling cells are most sensitive to radiation insult during transit through G2 and M phase, tumours displaying the accelerated cell cycle phenotype may also represent those that are most radiation-sensitive.

Traditional methods of cell cycle assessment in tumour samples, including flow cytometry, have limitations. Clinical samples are often unsuitable for such techniques, in part due to fixation artefacts, inadequate amounts of tissue and interpretation difficulties due to contaminating stromal/benign cell populations (Williams and Stoeber 2007). The presence of cells derived from other contaminating tissues, such as blood vessels and lymphoid follicles, also complicates the analysis by adding large numbers of cells with additional complex cell cycle kinetics. By contrast, multiparameter analysis of G1/S cell cycle regulators can be applied directly to paraffin wax-embedded tissue sections using relatively simple immunohistochemical methods; thereby providing a detailed characterisation of tumour cell cycle state. Prognostic algorithms for many tumour types

include a crude measure of their proliferative state, often based on mitotic index and/or Ki67 count (e.g. Nottingham Prognostic Index for breast cancer, Federation Nationale des Centres de Lutte le Cancer grading system for soft tissue sarcoma) (Elston 2002; Coindre 2006). Notably, many of the neoadjuvant and adjuvant chemotherapeutic interventions approved for clinical use include agents targeting either replicating cells (in S phase) or dividing cells (M phase), and will therefore only be effective against cells progressing through the cell cycle.

Interestingly, in PeScc the majority of tumours appear to exist in a "G1-arrested" state. This is characterised by an observed increase in the number of cells entering G1 phase (high 'Ki67 - geminin' score) but failing to progress through the G1/S transition (low geminin) with prolonged length of G1 phase (low geminin/Ki67 ratio). Proliferating cells with a short G1 phase will approximate to a geminin/Ki67 ratio of ~1, whereas cells with a prolonged G1 phase will approximate to a ratio closer to 0 (section 1.4.4.3). In contrast, a subset of tumours may exhibit rapid growth potential; demonstrating high Mcm2 and geminin levels which are indicative of accelerated cell dynamics and a shortened G1 phase. These results point to a clear implication on the efficacy of traditional chemotherapy agents in this tumour type given this multiparameter analysis approach. Often cytostatic and cytotoxic drugs are used in combination to help circumvent a lack of effect but this occurs at the expense of unacceptable levels of toxicity to patients (hair loss, gastrointestinal disturbance, mucositits, neutropenic sepsis and death). Adopting a multiparametric approach (Figure 4.10) to analyse tumour cell kinetics and phase specific targeting; may improve therapeutic validation and stratify patients to radical chemotherapy more appropriately, thereby avoiding a non-targeted style approach which

Figure 4.10

is often conducted through clinical desperation and harbour little chance of therapeutic success.

The inverse relationship between RLF expression and differentiation status recapitulates the findings witnessed in the *in vitro* HL60 monocyte/macrophage differentiation model system (Musahl et al. 1998; Barkley et al. 2007) and has been noted in several other malignancies (Going et al. 2002; Wharton et al. 2004; Kulkarni et al. 2007; Williams and Stoeber 2012). This relationship reflects the mutually antagonistic circuits that control cell proliferation and differentiation in human cells and highlights the potential clinical utility of RLFs for improving current tumour grading systems. These data show that analysis of RLFs alongside the ploidy status in primary biopsy material from PeScc can provide important additional prognostic information and identify those tumours with an aggressive cell cycle phenotype, the latter characterized by an increased growth fraction [i.e. an increase in the numbers of cells traversing G1 (Ki67-geminin score) and S-G2-M phases (geminin LI)] and accelerated cell cycle transit (geminin/Ki67 ratio). Notably, the aggressive tumour cell cycle phenotype is linked to general pathological features of aggressive disease, namely increasing: tumour size, stage, and depth of invasion as well as to morphologic subtypes associated with an adverse prognosis (e.g. basaloid). In the next chapter, I link this aggressive cell cycle phenotype to tumour ploidy status, suggesting that dysregulation of the DNA replication licensing pathway and cell cycle machinery is linked to the development of aneuploidy in PeScc.

The importance of RLF expression as a prognostic factor alongside conventional pathological grading and staging systems, in overall survival of men with PeScc, will

be investigated further in the subsequent chapters. There appear to be a number of promising translational indications with regards to diagnostic, prognostic and therapeutic potential for patients with PeScc. Important information can be retrieved from cytological or biopsy specimens through to cell cycle phase analysis and monitoring response to treatments.

4.5. Conclusion

In summary, using highly specific antibodies against Mcm2, geminin and the conventional pan-cycle marker Ki67, I have shown that RLF expression is tightly regulated during normal proliferation-differentiation processes. In normal penile squamous epithelium there is tight control of RLF expression and sharp downregulation of RLFs as cells withdraw from the cell division cycle to a terminally differentiated state. Through, these findings it is possible to identify cell populations residing in both in- and out-of-cycle states within a normal tissue system. These data recapitulate the findings observed in the *in vitro* HL60 monocyte/macrophage differentiation model system. In malignant transformation the replication licensing pathway clearly becomes dysregulated with grossly aberrant processes involved in advanced anaplastic growth. There is marked increase in RLF expression with increasing anaplasia and I have illustrated a strong correlation between conventional grading systems with increased cell cycle kinetics and phase-specific analysis. The information presented in this chapter identifies novel aspects to PeScc biology which provides an intriguing opportunity to develop novel prognostic and therapeutic modalities for these patients. In Chapter 5, I explore the role of RLFs in linking the development of anaplasia and genomic instability with advanced tumour states.

CHAPTER FIVE

REPLICATION LICENSING FACTORS CORRELATE WITH GENOMIC INSTABILITY REFLECTING AGGRESSIVE DISEASE PATTERNS

5.1 Introduction

5.2 Materials and Methods

5.3 Results

5.3.1 Dysregulation of the licensing machinery is linked to the development of genomic instability in PeScc

5.3.2 DNA licensing factors are dysregulated during progression from indolent to aggressive disease

5.4 Discussion

5.5 Conclusion

CHAPTER FIVE

REPLICATION LICENSING FACTORS CORRELATE WITH GENOMIC INSTABILITY REFLECTING AGGRESSIVE DISEASE PATTERNS

5.1. Introduction

Penile cancer represents a heterogeneous pathological process which may manifest with a variety of clinical symptoms and signs. The absolute aetiology of this rare, genitourinary malignancy remains undetermined; however the hypothesis that tumorigenesis is multifactorial appears justified (Rubin 2001; Kayes 2007; Prowse 2008; Andersson 2008). For clinicians, the identification of aggressive disease patterns is crucial in helping to stratify patients to specific treatment and surveillance options. This relies on accurate interpretation of important prognostic clinical/pathological variables which have now been identified through recent studies (Guimaraes et al. 2009; Cubilla 2009). A number of factors have been associated with aggressive disease states and guidelines based on the primary tumour grade and local stage have been published (Pizzocaro et al. 2010).

However, identifying aggressive PeScc can be difficult using traditional clincopathological markers alone as highlighted in chapter 1 (Section 1.5.2 & 1.5.3; Figures 1.7, 1.8 & 1.9). Advanced PeScc can be defined as: all locally advanced tumours (pT3 or pT4), lymph node positivity (pN1 to pN3) or the presence of

metastatic dissemination (M1). Additionally, it is essential to identify tumours with aggressive biological intent earlier and thereby increase the chances of cure and/or disease control for these patients. Finally, identification of novel molecular targets and improvements in the understanding of why current chemotherapeutic agents are generally ineffective in advanced PeScc is urgently required.

In section 1.5.4, I proposed a putative model for tumour progression in PeScc (Figure 1.10), based on the molecular biological evidence for this rare tumour type available in the current literature. Broadly, tumorigenesis appears to follow either HPV dependent or HPV independent pathways with invasive carcinomas developing from *in situ* neoplastic lesions or *de novo*. The multi-step Vogelstein-Fearon model for colorectal cancer (Fearon and Vogelstein 1990) appears to share a similar platform of sequential genetic and epigenetic mutations leading to invasive cancer. The distinct steps in this model include: initiation, promotion and progression. Therefore, it is possible to establish the successive genetic changes necessary to result in progression from an early cancer to an advanced and aggressive phenotype (i.e. anaplastic & higher stage with or without metastatic disease). Further work is required into the molecular mechanisms underpinning these biological events which tightly define each of these stages in PeScc. In this chapter, I aim to describe some of the core events in early and late tumorigenesis by analysing the dysregulation of the DNA replication licensing system and linking these data to the presence of chromosomal instability within these penile tumours.

The development of chromosomal instability is linked to the pathogenesis and progression of multiple tumour types (Deliveliotis 2003; Raatz 2004; Bantis 2005;

Deliveliotis 2005; El-Rayes 2005; Pinto 2005; Saiz-Bustillo 2005; Kasprzyk 2006; Araujo 2007; Santagostino 2007; Susini 2007; Yildirim-Assaf 2007; Pinto 2008; Susini 2011); however, the debate as to whether aneuploidy is a cause or consequence of cancer remains unresolved (Rajagopalan 2004). In PeScc, a number of karyotpyes have been described which match the chromosomal changes seen in other distinctive squamous carcinomas (Poetsch et al. 2007). Fundamentally, early tumourigenesis has been linked to deletions of the short arm of chromosomes 3 and 9. Despite, understanding this basic cytogenetic information, it is currently impossible to fully elucidate the role of penile intraepithelial neoplasia (PIN) in tumour development and progression in PeScc. Clearer evidence is now coming to light to suggest that PIN also develops in an HPV dependent and independent fashion and gives rise to specific invasive PeScc subtypes through proposed molecular pathways (Bleeker et al. 2009; Kayes 2012). Later tumourigenic processes are linked with greater number of alterations involving chromosomes 5, 8, 11, 18 and 21 (Poetsch et al. 2007). Tumours which behave in an indolent and unaggressive manner are characterised by their diploid cellular populations (Ornellas et al. 2000). Notably, a putative balance between distinct RLFs appears essential for maintaining chromosomal integrity and re-replication errors incurred if geminin and Cdt1 levels disassociate (Saxena 2005).

In previous chapters, I have described the validation of conventional clinicopathological features known to relate to aggressive oncogenic phenotypes in men with PeScc. I have also demonstrated that image cytometry offers an accurate and convenient method for analysing the DNA content of archival specimens and that aneuploidy is linked to locoregional disease. Furthermore, I have described the dysregulation of the DNA licensing system that occurs with neoplastic changes in

penile epithelium. In this chapter, I have sought to explore the link between these regulators of DNA replication with genomic instability as determined through ploidy studies. I have investigated the differences observed in RLF expression between diploid and aneuploid populations. Furthermore, I have analysed these factors alongside the tumour kinetics determined in chapter 4, in order to better understand the changes that occur between early and late tumorigenesis, reflecting a switch to more aggressive disease patterns.

5.2. Materials and methods

For Clinical data (2.2.1), Antibodies (2.3.1), Immunohistochemistry (2.3.3), Protein expression profile analysis (2.3.4), DNA ploidy analysis: image-based cytometry (2.5), Statistical methods (2.6) refer to Chapter Two.

5.3. Results

5.3.1. Dysregulation of the licensing machinery is linked to the development of genomic instability in PeScc

I sought to investigate the relationship between RLF expression (sensitive biomarkers of proliferation), anaplasia (arrested differentiation) and tumour DNA ploidy (genomic instability) in this study cohort of PeScc. This was achieved through the *in vivo* analysis of the expression profiles for these proteins determined by immunohistochemistry in 141 cases of PeScc and correlated with ploidy status determined by image cytometry. In dysplastic cases (n=14), the majority of lesions were diploid (65%). There does not appear to be a key signature in RLF expression that defines diploid from aneuploid cell populations in pre-malignant lesions (Table 5.1). Tumours showing increasing anaplasia also strongly exhibited aneuploidy

Table 5.1

($p<0.0001$); suggesting that arrested differentiation and aneuploidy are linked in PeScc. Moreover, the subset of aneuploid tumours was significantly linked to increased Mcm2, geminin and Ki67 expression ($p<0.01$) (Table 5.1). A decreased Mcm2/Ki67 ratio ($p=0.03$) and increased Ki67-geminin score ($p=0.01$) were also associated with increased DNA content. These data indicate an increased proportion of cycling cells in aneuploid tumours compared with diploid tumours. These findings support a hypothesis that aneuploid cancers are associated with a hyperproliferative state when compared with diploid tumours in PeScc.

5.3.2. DNA licensing factors are dysregulated during progression from indolent to aggressive disease

In order to explore the associations between aberrant DNA replication licensing and aneuploidy within aggressive disease states, I analysed the expression profiles for these biomarkers against the pathological stage of the tumours determined by the 2004 TMN staging system. Additionally, I analysed the correlation of RLF expression and DNA content with standard histopathological features of the primary tumour (Tables 5.2a & 5.2b). Aberrant RLF expression and aneuploidy are tightly correlated with increasing anaplasia as detailed in sections 3.3 and 4.3 Furthermore, a significant association between increased biomarker expression and geminin/Ki67 ratio with increasing tumour size and tumour subtype was observed. Previous research has shown that verrucous and warty tumours behave in a biologically indolent fashion with low risk for disease progression (Seixas et al. 1994) compared to aggressive variants, such as basaloid tumours which are associated with poorer clinical outcomes (Cubilla 2009). The innocuous clinical course observed with low risk tumours is reflected by the decreased growth potential and stable cell cycle dynamics witnessed

when compared to more aggressive variants. As such, low risk tumours were associated with lower Ki67 and RLF expression with the lower geminin and Ki67-geminin scores indicating smaller numbers of cells transiting S-G2-M and G1 phase respectively when compared with aggressive basaloid tumours (Tables 5.2a & 5.2b).

The subtypes associated with poorer clinical outcome therefore displayed a more aggressive cell cycle phenotype. Elevated geminin expression and an increase in geminin/Ki67 ratio were significantly associated with increasing local tumour stage (geminin: p=0.05; geminin/Ki67: p=0.02) and increasing depth of invasion (geminin: p=0.02; geminin/Ki67: p=0.03) (Tables 5.2a & 5.2b). Higher tumour stage and increased depth of invasion appear to be features of aggressive cancers (Cubilla 2009; Pizzocaro et al. 2010). I have shown clearly that these tumours demonstrate an increased proliferative signature with aggressive tumours demonstrating accelerated cellular kinetics with shortened G1 phase times, as the geminin/Ki67 ratio approximates towards unity (see section 1.4.4.3 and 4.3.5). Interestingly, the positive correlation between geminin expression and increasing tumour anaplasia indicates that this licensing repressor does not behave as a tumour suppressor in PeScc and is in keeping with previous studies in other tumour types, which demonstrate the direct correlation between geminin expression and proliferation index (Wohlschlegel *et al* 2002; Eward *et al* 2004; Wharton *et al* 2004; Dudderidge *et al* 2005; Obermann *et al* 2005; Shetty *et al* 2005; Dudderidge *et al* 2007). Finally, I examined the critical associations between RLF expression, DNA content and the development of regional (inguinal and pelvic lymph nodes) and/or distant metastases. In univariate analysis, Mcm2 (P = 0.02), geminin (P = 0.02) and Ki67 (P = 0.03) expression were all significant predictors of nodal status (Table 5.3). As observed in section 3.3.3, tumour

grade (P = 0.02) and stage (P < 0.01), presence of vascular invasion (P = 0.04), and ploidy status (P = 0.03) were also predictive of nodal status; with poorly differentiated tumours [odds ratio (OR), 8.76; 95% CI, 1.80-42.73], high-stage disease (OR, 4.80; 95% CI, 1.81-12.74), presence of vascular invasion (OR, 2.66; 95% CI, 1.04-6.75), and aneuploidy (OR, 2.96; 95% CI, 1.09-8.01) closely linked to advanced disease with regional lymph node involvement (Table 3.3).

Multivariate analyses were used to construct a final model using these factors. After backward elimination, biomarker information (Mcm2, Ki67, or geminin LI), vascular invasion, and ploidy status were excluded from the final analysis. Tumour grade (P = 0.006) and local stage (P = 0.003) were independent predictors of positive lymph node disease (Table 3.4). The area under the receiver operating characteristic curve for this model was 0.78 (95% CI, 0.69-0.87). Although all biomarkers were predictive markers in univariate analysis, no single biomarker was a significant predictor after adjustment for grade and stage. This is due, in part, to the significant associations between biomarker LI with tumour grade and stage, making it difficult to separate their independent effects. Finally, on further analysis, I observed a significant association between aneuploid tumours (p=0.03) and a trend for tumours showing increased Mcm2 expression (p=0.09) with the development of distant metastases; thus reflecting late cancer progression. These striking outcomes are explored in greater detail in chapter 6 in combination with overall survival data.

Table 5.2a

Table 5.2b

Table 5.3

5.4. Discussion

Evolutionary studies of human solid tumours provide not only a model of tumour progression, but potentially the means to identify distinct biological elements that might predict, detect, diagnose and treat human malignancy. Many cancers proceed through definitive stages that offer excellent opportunities to alter the course of disease; however, the inherent complexity of neoplasia and unique attributes of different tumour types dictate a multidimensional approach to identifying and evaluating molecular markers that incorporates knowledge of the stages and critical pathways involved in initiation and progression. The DNA replication licensing system lies at the convergent point of upstream mitogenic signalling pathways (Williams and Stoeber 2007; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013). Through understanding how this system is dysregulated in early and late carcinogenesis, it may be possible to target specific intracellular components for improved diagnosis, prognosis and treatment in men with PeScc and provide critical target validation for novel agents acting as new cancer therapeutics (Williams et al. 1998; Freeman et al. 1999; Stoeber et al. 1999; Kodani et al. 2001; Stoeber et al. 2002; Going et al. 2002; Williams et al. 2004; Laskey 2005; Gonzalez et al. 2005; Scott et al. 2006; Williams and Stoeber 2007; Scarpini et al. 2008; Siddiqui et al. 2008; Ayaru et al. 2008; Kulkarni et al. 2009; Loddo et al. 2009; Rodriguez-Acebes et al. 2010; Dudderidge et al. 2010; Depuydt et al. 2011; Saeb-Parsy et al. 2012; Williams and Stoeber 2012; Kelly et al. 2012; Jackson, Laskey, and Coleman 2013).

The multi-step tumourigenesis model for colorectal cancer (Fearon and Vogelstein 1990) provides a basic template for the accumulation of genomic instability observed from cancer initiation through cancer progression. PeScc potentially represents a more

heterogeneous and diverse pathology which may share key mechanistic events with other crucial urogenital and gynaecological tumours; specifically squamous cell carcinoma of the vulva (Longpre et al. 2012) and adenocarcinoma of the uterine cervix (Grulich et al. 2010; Wattleworth 2011). It has been demonstrated in principle that invasive carcinomas of the penis may arise from pre-malignant lesions or *de novo* and that the underlying forces driving carcinogenesis in these groups are remote from each other and possibly linked to HPV infection (Bleeker et al. 2009; Kayes 2012; Velazquez, Chaux, and Cubilla 2012).

I sought to understand the relationship of origin licensing with genomic instability in penile intraepithelial neoplasia (PeIN) and invasive PeScc. The DNA replication licensing system appears to be highly dysregulated in pre-malignant lesions characterised by a high proliferative signature (i.e. high expression Mcm2; Ki67; geminin) in the context with full thickness dysplasia. Here, it is observed that these early events in PeScc carcinogenesis are usually associated with diploid DNA content of the dysplastic cells. This suggests that the replication licensing system is dysregulated at an early stage in PeScc, as observed in other tumour types (Williams et al. 1998; Freeman et al. 1999; Stoeber et al. 1999; Kodani et al. 2001; Going et al. 2002; Stoeber et al. 2002; Williams et al. 2004; Gonzalez et al. 2005; Scott et al. 2006; Williams and Stoeber 2007; Ayaru et al. 2008; Scarpini et al. 2008; Dudderidge et al. 2010; Kelly et al. 2012; Saeb-Parsy et al. 2012; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013). Interestingly, it has been reported that between 5-33% of PeIN will progress to invasive carcinoma (Bleeker et al. 2009) which may reflect the proportion of lesions with genomic instability in this dataset (35%). An alternative hypothesis could suggest that these aneuploid PeIN lesions may represent

a highly unstable epithelial precursor that may simply progress more rapidly if left untreated.

Conversely, poorly differentiated, invasive carcinomas are significantly linked to elevated expression of all RLFs and are commonly aneuploid (Figure 5.1). Furthermore, aneuploid tumours are associated with a decreased Mcm2/Ki67 ratio ($P = 0.03$); and increased Ki67-geminin score ($P = 0.01$) indicating an increased proportion of actively cycling tumour cells in aneuploid tumours compared with diploid tumours. Importantly, these alterations in cell cycle regulation correlate with late carcinogenic events including: increasing tumour size, local invasion and development of regional and distant metastases. Geminin levels appear to be crucial in this process, promoting cell cycle progression in conjunction with dysregulation of normal cellular checkpoints. Several studies have reported the strong link between increased geminin expression and aggressive tumour factors (Shetty et al. 2005; Shrestha et al. 2007; Loddo et al. 2009; Nishihara et al. 2009; Kapoor 2012; Jackson, Laskey, and Coleman 2013). The proteins involved in DNA replication licensing appear to become increasingly dysregulated during the progression from low-grade, diploid tumours to aggressive high-grade, aneuploid tumours (Kulkarni et al. 2007; Loddo et al. 2009). This supports the hypothesis that not only may these proteins become dysregulated during the initiation of malignant potential but also that their continued aberrant regulation reflects tumour progression.

Figure 5.1

These data highlight an intriguing connection between chromosome replication initiation and polyploidy in cancer development and progression. The replication licensing pathway is integral to early tumour formation and is markedly dysregulated in late PeScc carcinogenesis as shown by the close correlation to aneuploidy progenies. Figure 5.1 attempts to further describe and develop the putative initiation and progression model originally discussed in chapter 1 (Figure 1.10). The role of HPV infection is critical to tumorigenesis for a subset of penile cancers (approximately 50%) (Della et al. 1992). This is important information for current WHO vaccination programmes (Crosignani et al. 2013) as the overall impact on the incidence of this rare tumour will remain small (Anderson 2012). The new model proposed in this section provides new, insight to distinct pathways involving potentially attractive molecular targets for novel therapeutic agents or prognostics tests.

The targeting of upstream growth signalling pathways is often constrained by pathway redundancy (Williams and Stoeber 1999) or the development of growth-independent (autonomous) cancer cell cycles (Williams and Stoeber 2007). Efficacy can be compromised through a variety of mechanisms, e.g. through overexpression of alternative receptor tyrosine kinases or development of new signalling pathways (Sierra, Cepero, and Giordano 2010). Therapeutic targeting of the DNA replication initiation machinery, which lies at the convergence point of growth signalling networks, is now emerging as a new concept promising to overcome the limitation of targeting more upstream pathways. Potent cancer cell-specific killing has been demonstrated in preclinical models after inhibition of origin licensing (Shreeram et al. 2002) or, alternatively, origin activation through targeting Cdc7 kinase (Montagnoli et

al. 2004; Montagnoli et al. 2008; Kulkarni et al. 2009; Rodriguez-Acebes et al. 2010). Several studies have shown that following impairment of the DNA replication initiation machinery, normal cells arrest at the G1 –S boundary with unreplicated DNA, elevated p53 levels and induction of CDKI p21 (Shreeram et al. 2002; Montagnoli et al. 2004; Rodriguez-Acebes et al. 2010). This putative cell cycle checkpoint appears critically dependent on several tumour suppressor proteins, including p53, p21, Dkk3, ARF, Hdm2, FoxO3a, p15, p27 and RB (Tudzarova et al. 2010). This suggests that inactivating mutations in checkpoint proteins, such as Rb and p53 which are commonly seen in penile cancer; will render these cells exquisitely sensitive to anti-cancer agents targeting the DNA replication initiation machinery (Tudzarova et al. 2010).

Alternatively, translation of these results to clinical practice may represent an opportunity to effect changes to current grading systems (Broders AC. 1921; Maiche 1991) given the highly significant association between these cell cycle regulators and tumour grade. These results have been consolidated in several other malignancies (Sudbo 2001; Wharton 2004; Shetty et al. 2005; Graafland 2011). "Molecular staging" based on a panel of molecular markers can complement current clinicopathological staging to accurately indicate the risk of disease progression through multi-parameter analysis exploring the cell cycle progression in PeScc. These findings are in keeping with similar analyses of RLFs in prostate, bladder, lung, breast and renal cell cancer (Freeman et al. 1999; Kodani et al. 2001; Meng 2001; Gonzalez 2003; Wharton 2004; Dudderidge 2005; Gonzalez et al. 2005; Shetty et al. 2005; Dudderidge 2007; Kulkarni et al. 2007; Williams and Stoeber 2007; Loddo et al. 2009; Williams and Stoeber 2012; Jackson, Laskey, and Coleman 2013).

Identification of specific molecular targets that may be more amenable to specific therapies would clearly be advantageous. These novel staging systems may then be used for administering targeted therapy, which can be individually tailored to meet a patient's molecular profile and projected response, thus decreasing morbidity (Figure 5.2).

5.5. Conclusion

The data presented in this chapter provides important evidence supporting a link between aberrant regulation of the replication licensing system and the development of genomic instability in PeScc. As discussed in chapter 4, cell cycle phase analysis of these tumours helps to identify differential tumour cell populations which define early and later carcinogenic events, exemplified here by correlation to standard clinicopathological factors linked to advanced clinical disease. Expanding on the putative biological mechanisms that appear to drive tumour progression in PeScc, namely hyperproliferation and genomic instability; I subsequently explore whether these correlates provide realistic translation value for predicting survival in these men. In chapter 6, I link the RLF expression and DNA ploidy of these tumours to survival data and test the prognostic power of a new staging system to discriminate a patient’s risk for progression based on the pathological and molecular markers studied to date.

Figure 5.2

CHAPTER SIX

PROGNOSTIC INFORMATION FOR DNA REPLICATION LICENSING FACTORS AND ANEUPLOIDY IN PENILE CARCINOMA

CHAPTER SIX

PROGNOSTIC INFORMATION FOR DNA REPLICATION LICENSING FACTORS AND ANEUPLOIDY IN PENILE CARCINOMA

6.1. Introduction

Advances in genomics, proteomics and molecular pathology have generated many candidate biomarkers with potential clinical value across different tumour types. However, translation from 'bench to bedside' outside of the research setting has proved difficult. There have been a lack of reliable, prospective studies and in many cases primary data is often statistically over-fitted leading to a lack of reproducibility. Five conceptual phases of biomarker development (Rifai 2006) have been proposed (Pepe 2001) :

1. preclinical exploratory
2. clinical assay and validation
3. retrospective longitudinal
4. prospective screening
5. cancer control

The process is fraught with difficulties and most candidate markers are still in the early phases of development. A new cancer biomarker under development is likely to have already encountered one or more of the following fatal features encountered by

prior markers: lack of clinical significance, hidden structure in the source data, a technically inadequate assay, inappropriate statistical methods, unmanageable domination of the data by normal variation, implausibility, deficiencies in the studied population or in the investigator system and its disproof or abandonment for cause by others (Rifai 2006).

These processes are focussed on developing modern, personalised, tumour diagnostics and prognostics through combinatorial approaches of multiple biomarkers. Hopefully, this will obviate the heterogeneity seen in most tumours and guide diagnosis, treatment and follow up, especially in equivocal cases. Hence, future biomarkers should be able to reflect prognostic information set against disease free and overall survival data; as well as a predictive value to assess appropriate treatment responses (Williams 2007) .

The formal TNM staging system is based almost exclusively on the anatomical extent of disease, which is assessed using a combination of tumour size or depth (T), lymph node spread (N), and presence or absence of metastases (M). Since its inception in 1953, the TNM system has provided a standardized, anatomical basis for staging different malignancies (Denoix 1953). Less often, tumour grade, histological subtype or patient age has been added to TNM staging. Currently, none of the FDA approved biomarkers have been incorporated into current staging systems. Each anatomical site has its own histological grading system, designed to classify malignancies by degree of differentiation.

Low-grade, well-differentiated tumours are usually less aggressive and more favourable in prognosis than high-grade tumours, which tend to grow faster and metastasize earlier. However, tumour grade is only included in formal TNM staging, when intimately linked to prognosis, as it is for soft-tissue sarcomas, prostate cancer and primary brain malignancies (Wittekind 2002) . Assignment of grade is inherently subjective and dependent on the skill and experience of the reviewing pathologist. Biomarker expression can reliably score tumours according pathologist reported grades with additional potential for semi-automotive quantification which will permit fast and accurate diagnostics.

Targeted therapies, which include monoclonal antibodies and small molecule inhibitors, have significantly changed the treatment of cancer over the past decade. Alongside standard chemotherapy (Table 6.1), these drugs are now more commonplace in the treatment of many common malignancies including: breast, colorectal, lung, and pancreatic cancers, as well as lymphoma, leukemia, and multiple myeloma (Table 6.2). However, these agents are effective only if their respective molecular markers are mutated or expressed at sufficient levels. The mechanisms of action and toxicities of targeted therapies differ from those of traditional cytotoxic chemotherapy. Targeted therapies are generally better tolerated than traditional chemotherapy but they are associated with several adverse effects such as acneiform rashes, cardiac dysfunction, thrombosis, hypertension, and proteinuria (de, Jr. 2006) . Small molecule inhibitors are metabolized by cytochrome P450 enzymes and are subject to multiple drug interactions. The use of targeted therapy has markedly changed outcomes for some cancers and has revolutionized treatment. In other

instances, the degree of clinical benefit is more modest. Before the advent of targeted therapies, patients with metastatic renal cell carcinoma treated with cytokines showed a median survival of 10 months (Motzer 1999) . However, newer agents including; sunitinib, sorafenib, and pazopanib, demonstrate efficacy and constitute the comparator arm in several ongoing studies. In patients with advanced renal cell carcinoma, the initial data for sunitinib against standard therapy in the form of immunotherapy (alpha-interferon) showed a real but modest increase in median survival from 21.8 to 26.4 weeks (Motzer 2007; Motzer 2009) .

The process of PeScc carcinogenesis appears to be driven by a core number of oncogenic mutations which far out-strip the rudimentary algorithm proposed in sections 1.5.5 and 5.4. Hence, these epithelial tumours can present with variable clinical phenotypes underpinned by a diverse genological heritage and equivocal biological behaviour. The complexity of targeting upstream growth signalling pathways limits current therapeutic and prognostic modelling. In order to minimise the variability observed through the heterogeneous biology of most tumours, including PeScc, targeting the ubiquitously up-regulated cell proliferation powered by dysregulation of the cell cycle machinery appears to represent a core mechanism (Hanahan and Weinberg 2000; Evan and Vousden 2001; Jackson, Laskey, and Coleman 2013; Williams 2012). Ultimately, any oncogenic events upstream of the cell cycle machinery will be mirrored in the extent of its dysregulation.

In this final chapter, I explore the early clinical data evaluating the impact of RLFs as novel prognostic indicators of disease progression and outcome in patients with PeScc. Using the validated dataset presented in this thesis, I will aim to correlate RLF expression with traditional clinicopathological variables and ploidy status against

Table 6.1

Table 6.2

overall survival. Furthermore, using contemporary statistical techniques, I construct an innovative risk profile for these tumours to help stratify patients more succinctly into discrete categories in order to aid adjuvant therapies and surveillance programmes.

6.2. Materials and methods

For Clinical data (2.2.1), Antibodies (2.3.1), Immunohistochemistry (2.3.2), Protein expression profile analysis (2.3.4), DNA ploidy analysis: image-based cytometry (2.5), Statistical methods (2.6) refer to Chapter Two.

6.3. Results

6.3.1. RLF expression, DNA content, tumour characteristics and overall survival

I investigated the survival time for patients with PeScc using Cox's proportional hazards model. The study group for the analysis included 118 men with a recorded survival time. Of these, 26 patients were dead (22%) and 92 were alive (78%) at the time of analysis. Univariate analysis (Table 6.3) showed that Mcm2 ($P = 0.02$), Ki67 ($P = 0.04$) expression and Ki67-geminin score ($P = 0.03$) were all significantly associated with overall survival. Age ($P = 0.002$), tumour stage ($P = 0.02$), depth of invasion ($P < 0.0001$), tumour multifocality ($P = 0.002$), vascular invasion ($P = 0.04$), and ploidy status ($P = 0.008$) were also predictive of overall survival outcomes. Advanced age [hazard ratio (HR) 1.05 per year; 95% CI, 1.02-1.1], higher-stage tumours (HR 3.89; 95% CI, 1.52-9.99), multifocal tumours (HR 3.69; 95% CI, 1.63-8.32), increased depth of invasion (HR 1.045; 95% CI, 1.025-1.064), presence of vascular invasion (HR 2.23; 95% CI, 1.02-4.87), and aneuploidy (HR 4.28; 95% CI,

1.46-12.58) were associated with a significantly shorter overall survival time. It was also noted that lymph node status (negative or positive) was an excellent prognostic factor on univariate analysis (HR, 11.07; 95% CI, 3.74-32.71) in keeping with previous reports. Notably, tumour grade failed to predict outcome in this series (P = 0.28) emphasizing the limitations with current grading systems.

Multivariate analyses were used to construct a final model involving 84 patients (76 Alive; 18 Dead). Backward elimination resulted in the exclusion of biomarker information (Mcm2, Ki67, or geminin LI), vascular invasion, local tumour stage, and depth of invasion from the final model. However, age (P = 0.004), positive lymph node status (P < 0.001), tumour multifocality (P = 0.002), and aneuploidy (P = 0.03) were identified as independent predictors of overall survival (Table 6.4).

6.3.2. Risk profiling in penile cancer

I quantified the discriminatory ability of this model using Harrell's c-index (FE Harrell, Regression Modeling Strategies, Springer, 2001). This is analogous to the receiver operating characteristics (ROC) area and gives the probability that a randomly selected pair of patients has concordant predictions and survival times (i.e. the patient with the best prognosis also has the longer observed survival time). The c-index takes values between 0.5 (random predictions) to 1 (perfect concordance). In isolation, these predictors have c-index values of 0.77 (lymph node status), 0.68 (age), 0.65 (aneuploidy), and 0.64 (multifocality). The multivariable model has a Harrell's c-index of 0.88. Kaplan-Meier survival curves show the survival advantages for standard clincopathological variables (Figure 6.1 to 6.3), biomarker expression

TaBLE 6.3

Table 6.4

(Figure 6.4) and ploidy status (Figure 6.5). In combination, these data suggest that the implementation of a 'semi-automotive' process to analyse tumours for aneuploid DNA content and increased RLF expression (Mcm2 and/or geminin) can provide important staging and prognostic information at an earlier stage of the disease and treatment process in these men. This further supports the rationale for developing a multiparameter test using this unique panel of biomarkers as previously outlined in section 5.4. This should allow improved prognostication compared to current standard histopathological assessment and can be performed on biopsy material alone in principle. To illustrate the predicted survival based of this model, the patients were split into tertile groups and labelled as low, medium and high risk (Table 6.4). The model has the form:

$$\textbf{Prognostic Index (PI)} = [2.14 \times \text{Node } (\textit{Positive})] + [1.58 \times \text{Extent } (\textit{Multifocal})] + [1.43 \times \text{Ploidy } (\textit{Aneuploid})] + [0.0551 \times (\textit{Age})]$$

For an individual patient it is possible to calculate their prognostic index (PI) and split the patients into tertile based on the PI score (low-risk: PI < 4.4, medium-risk: 4.4 < PI < 6.2, high-risk: PI > 6.2). Kaplan-Meier survival curves were constructed for these tertile risk groups derived by splitting the patients into equal-sized groups based on their predicted risk from the model (Table 6.5; Figure 6.6). Using this model, the overall survival rates observed at 3 years were 97%, 93%, and 27%, and 5-year survival figures of 97%, 72%, and 18% for low, moderate, and high risk men, respectively. This final risk profiling model will hopefully obviate the need for unnecessary surgery or radiological investigation in some patients, whilst highlighting high risk patients who may benefit from adjuvant therapies.

Figure 6.1

Figure 6.2

Figure 6.3

Figure 6.4

Figure 6.5

Table 6.5

Figure 6.6

6.3. Discussion

In this study, I have assessed the utility of Mcm2 and geminin, alongside DNA ploidy status as novel biological predictors of outcome in men with PeScc. My results show that Mcm2 and geminin labeling indices in conjunction with aneuploidy are powerful prognostic indicators of locoregional metastasis and overall survival. Predictive nomograms for lymph node involvement and cancer-specific survival in PeScc using traditional histopathologic information alone have been developed but require prospective validation (Ficarra 2006; Kattan 2006) . In this study: Mcm2 and Ki67 LIs, Ki67-geminin score, age, tumour stage, depth of invasion, tumour extent/location, vascular invasion, lymph node status and ploidy status were all identified as predictors of overall survival; with lymph node status, tumour extent, and ploidy status identified as independent predictors of overall survival. Notably, I have shown that these parameters can be incorporated into a simple predictive model to stratify patients into high, intermediate, and low-risk groups for disease progression in PeScc. Crucially, the multivariable model suggests that low-risk patients are at minimal risk of disease progression compared with men assigned to moderate or high-risk groups. This is highlighted by overall survival rates at 3 years of 97%, 93%, and 27%, and 5-year survival figures of 97%, 72%, and 18%, for low, moderate, and high-risk men, respectively. This provides a potentially powerful approach for early treatment stratification of patients, with low-risk patients assigned to surveillance programs whilst targeting high-risk patients with radical surgical and adjuvant chemotherapeutic interventions. Further studies in additional patient cohorts are now warranted to confirm the predictive power of this model in the risk stratification of PeScc.

Inhibition of the DNA replication initiation machinery has been shown to provoke a cancer cell–specific apoptotic response as a result of the loss or impairment of a putative checkpoint for replication-competent origins during tumourigenesis (Rodriguez-Acebes 2010; Tudzarova 2010). Potent cancer cell-specific killing has been demonstrated in preclinical models after inhibition of origin licensing (Shreeram 2002) or, alternatively, origin activation through targeting Cdc7 kinase (Montagnoli 2004; Montagnoli 2008). Tumour cell specificity is thought to result from transformed cells entering S phase with inadequate numbers of competent origins to complete chromosomal replication. This results in an abortive S phase with incompletely and/or abnormally replicated DNA. Tumour cells with a functional intra-S phase checkpoint appear to undergo rapid death after replication fork stalling/collapse, whereas more transformed cancer cells appear to survive longer but eventually face mitotic catastrophe as a result of partially replicated chromosomes (Montagnoli 2004; Montagnoli 2008; Shreeram 2002). In striking contrast, normal cells avoid entering S phase with a reduced number of replication-competent origins by engaging a recently described cell cycle checkpoint, the 'origin activation checkpoint'. Several studies have shown that following impairment of the DNA replication initiation machinery, normal cells arrest at the G_1–S boundary with unreplicated DNA, elevated p53 levels and induction of CDKI p21 (Montagnoli 2004; Shreeram 2002). We can therefore hypothesise that loss of the protective checkpoint mechanism through inactivating mutations in checkpoint proteins may render most common solid tumours sensitive to anti-cancer agents targeting the DNA replication initiation machinery.

In summary, I have shown that increasing dysregulation of the DNA replication licensing pathway is linked to emergence of an aggressive cell cycle phenotype that

affects the *in vivo* behaviour of this tumour type. The DNA replication licensing pathway therefore also seems to be a potentially attractive therapeutic target in PeScc. The rationale and potential for developing novel Cdc7 kinase inhibitors is discussed further in the final chapter.

6.4. Conclusion

In summary, it is apparent that several of the proteins I have investigated are of important prognostic value as biomarkers in PeScc. Patient stratification for radical and adjuvant treatments will be aided by adopting this novel multiparameter analysis. Furthermore, it may be possible to implement these diagnostic and prognostic strategies at an earlier stage than current grading and staging algorithms. Finally, these data provide crucial target validation for developing novel small molecule therapeutics against the RLF machinery, as new and complimentary treatments in this highly complex malignancy.

CHAPTER SEVEN

CONCLUDING REMARKS

CHAPTER SEVEN

CONCLUDING REMARKS

7.1. Conclusions

PeScc is a rare and unusual genitourinary malignancy with a highly complex and heterogeneous heritage, reflected in the poorer overall outcomes and lack of efficacious first/second line chemotherapeutics in the management of advanced disease states (Hakenberg 2012). Classically, the progression to metastatic disease follows well-defined patterns of dissemination involving locoregional lymphatic spread to eventual distant metastases. It is not unusual for men to present with locally destructive lesions and the presence of inguinal deposits. Current surgical management strategies are often physically and psychologically debilitating. In contrast, men presenting with earlier disease offer a diagnostic and therapeutic dilemma, as current staging modalities lack the necessary sensitivity and specificity to stratify patients accurately to surveillance programmes versus adjuvant surgical and therapeutic regimes. In order to impact on the poor survival rates seen in advanced PeScc, it is crucial to understand the mechanisms involved in malignant progression. This would enable prediction of those tumours at high risk of progression or relapse at diagnosis and identify those likely to benefit from specific therapies; thereby offering the opportunity to tailor management. The goal of achieving such personalised treatment has been the major focus of cancer research, across a broad range of malignancies. However to date, such success remains elusive in PeScc, not least due

to the rarity of the condition and also the complexity of the molecular events which occur during the transition from benign to malignant disease.

Notably, expression arrays in a broad range of malignancies have identified 'proliferation signatures', genes whose expression correlates with tumour grade, cell cycle status and doubling times (Ross 2000). This proliferation signature is one of the most prominent gene-expression patterns observed in tumour datasets and includes many cell cycle regulated genes, several of which have been shown to have a role in carcinogenesis. This is unsurprising when one considers the importance of the cell cycle machinery as a convergence point for upstream oncogenic signalling pathways (Evan 2001; Williams 2007; Jackson, Laskey, and Coleman 2013; Williams 2012). In the work of this thesis, I have focused on key G1/S cell cycle regulatory molecules which govern the DNA replication licensing system. Importantly, not only have these proteins been identified in several tumour types, including large-scale microarray datasets (Rhodes 2004; Whitfield 2002), as potential prognostic indicators, they are also attractive therapeutic targets and are therefore the focus of current drug development programmes (Jackson, Laskey, and Coleman 2013; Williams 2012). However there is a paucity of information regarding the role of these regulatory proteins in PeScc. In the work of this thesis, I have shown that there is aberrant regulation of the RLFs in PeScc. The broad utility of these proteins at various stages in the management of cancer, from population screening to tumour relapse, highlights several interesting prognostic and therapeutic avenues which arise from this work (Jackson, Laskey, and Coleman 2013; Williams 2012).

Firstly, they may be used as proliferation markers with potential for further

improvements in the current grading system and as molecular markers of tumour stage allowing refinement of present surgical and radiological methods (Chapters 3 & 4). Moreover, the observation that their expression does not fully distinguish between tumour grades demonstrates that traditional clinicopathological parameters do not always allow prediction of therapeutic response, supporting the concept of co-evolution of biomarker and individualised targeted therapy. This has attractive implications for diagnosing and/or monitoring new and recurrent penile epithelial lesions without the need for repeated biopsy. This approach further exploits the surface sampling principles observed in other tumour types (Ayaru 2008; Dudderidge 2010; Going 2002; Jackson, Laskey, and Coleman 2013; Kodani 2001; Scott 2006; Stoeber 2002; Williams 1998; Williams 2004; Williams 2007; Williams 2012) and which now form the basis of several, large FDA clinical trials (Depuydt 2011; Tambouret 2008). Furthermore, it may be possible to utilise the important prognostic information delivered through analysis of these biomarkers to deliver more appropriate treatments for men at an earlier stage of their clinical management (i.e. on initial biopsy material). Hopefully, this will allow more men to receive effective adjuvant treatments without delay; whilst sparing low risk men from the notable side-effects of contemporary surgical and chemotherapeutic treatments. Secondly, the multiparameter analysis of RLFs provides insight into the cell cycle state of individual tumour samples (Chapter 4, 5 & 6). This form of analysis may be used in predictive testing for both cell-cycle phase specific chemotherapeutic drugs and novel small molecules targeting the cell cycle machinery or upstream growth signal transduction pathways that accelerate cell cycle progression. For example, tumours demonstrating high Mcm2 and Ki67 levels with low geminin co-expression, thus indicating an arrested or prolonged G1 phase (observed in PeScc), may be less likely to respond to

S or G2/M cell cycle phase-specific drugs.

In this work, I have also demonstrated that there is an intricate link between dysregulation of these cell cycle regulatory proteins and the development of genomic instability and advanced disease states in PeScc (Chapter 5). Ploidy status provides important independent prognostic information but additionally the observed dysregulation of RLF expression presents further evidence that this pathway is a potentially important novel therapeutic target. Notably, in light of its kinase activity and amenability to small molecule inhibition, Cdc 7 has been shown to be a promising "anticancer" molecular switch (Montagnoli 2004; Williams 2012). Cdc7 is a highly conserved serine/threonine protein kinase and a core component of the licensing machinery required for origin unwinding of the DNA, and recruitment of DNA polymerases required for DNA synthesis (Tudzarova 2010; Williams 2012). Evidence suggests that in untransformed cells, inhibition of origin firing triggers the putative "licensing checkpoint", leading to a block to DNA replication initiation and stable G1 arrest (Montagnoli 2004; Rodriguez-Acebes 2010). It has been recently shown that this checkpoint response is critically dependent on several common tumour suppressor genes including p53, p21, Dkk3, ARF,Hdm2, FoxO3a, p15, p27 and RB (Figure 7.1) (Tudzarova 2010). The checkpoint response is dependent on three axes coordinated through the transcription factor FoxO3a. In arrested cells, FoxO3a activates the ARF --| Hdm2 --| p53 → p21 pathway and mediates p15(INK4B) upregulation; p53 in turn activates expression of the Wnt/β-catenin signalling antagonist Dkk3, leading to Myc and cyclin D1 downregulation. The resulting loss of CDK activity inactivates the Rb-E2F pathway and overrides the G1-S transcriptional programme. Subsequently, E2F cannot then drive expression of replication factors:

Figure 7.1

Mcm2-7, Mcm10, geminin, Dbf4, Cdt1 and Cdc6 (Tudzarova 2010; Williams 2012); so origins become unlicensed, thus enforcing the observed G1 arrest. In contrast to untransformed cells, transformed cells are unable to trigger a checkpoint response due to inactivation of tumour suppressor genes. This results in transformed cells entering S phase with inadequate numbers of competent origins to complete chromosomal replication (Tudzarova 2010; Williams 2012). Insufficient Cdc7 availability required to trigger the 30,000 origins necessary to complete S phase in 6-8 hours, leads to fork stalling and collapse of replication forks, the induction of double-strand breaks and apoptosis in these cancer cells (Montagnoli 2008; Swords 2010). This results in an abortive S phase with incompletely and/or abnormally replicated DNA. Tumour cells with a functional intra-S phase checkpoint appear to undergo rapid death after replication fork stalling/collapse, whereas more transformed cancer cells appear to survive longer but eventually face mitotic catastrophe as a result of partially replicated chromosomes. Notably the checkpoint is critically dependent on both Rb and P53 function. Importantly, these two tumour suppressor proteins are dysregulated in PeScc by HPV E6 and E7 (Figure 5.1); therefore PeScc represents an ideal tumour type to treat with a Cdc7 inhibitor and indeed mirrors the situation in ovarian cancer in which p53 and Rb are both inactivated and in which potent tumour cell killing has been observed following Cdc7 inhibition (Kulkarni 2009). The availability of Cdc7 small molecular inhibitors (SMI) now provides an exciting opportunity for this novel treatment paradigm to be tested (Koltun 2012; Natoni 2011; Swords 2010; Woods 2012); (CRT: Cdc7 marketing information – Appendix F).

In conclusion, the work presented in this thesis provides a strong rationale for exploitation of the DNA replication licensing system alongside ploidy status as novel

prognostic/predictive biomarkers and molecular therapeutic targets in PeScc. The findings discussed above pave the way for future experimental work towards two major goals. Firstly, the prognostic value of these biomarkers highlights the need for large well-designed prospective, multicentre, international randomised controlled trials in PeScc, which can confirm their utility in predicting patient survival and tumour response to specific chemotherapeutic and biological therapies. The information obtained from such prospective studies could subsequently be used to tailor treatment options for patients based on the underlying biology of their tumour. This form of predictive testing would be invaluable in assisting decisions regarding the usage of cytotoxic drugs, with their associated toxicities, and biological agents in patients who may not benefit from such treatments.

Secondly, the identification of Cdc7 kinase as a promising new therapeutic target may offer new treatment options in a variety of solid tumours but particularly in PeScc which is driven by HPV-dependent dysregulation of Rb and P53 pathways in certain patients. Importantly by targeting Cdc7 there appears to be minimal effects on normal cells, which has not been the case with the majority of other approaches to inhibition of the cell cycle. Thus, this approach potentially circumvents the cytotoxic side effects seen with traditional chemotherapeutic drugs, for example: hair loss, infertility and suppression of self-renewing tissues such as the haematopoietic and gastrointestinal systems. Low blood counts and gut-related symptoms, for example gastrointestinal haemorrhage, are rate-limiting toxicities for conventional cytotoxic agents. By contrast, cyclical treatment with a Cdc7 kinase inhibitor should have minimal effects on normal tissues and thus be associated with significantly fewer side effects. The main aim for future studies will therefore be to confirm the safety, efficacy and

tolerability of such an agent and assess translational molecular-based endpoints alongside survival and quality of life outcomes, thereby moving one step closer to the goal of personalised therapy in PeScc.

CHAPTER EIGHT

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APPENDIX A

Summary of clinical database

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
1	17/01/1992	33	Lost to follow up	Lost to follow up	Cis	WLE	Cis	Tis	Nx	0
2	01/06/2001	74	ALIVE	58.3	Nos	Glansectomy	1	1	0	0
3	05/12/1991	78	DEAD	14.5	Nos	Total Penectomy	3	3	3	Mx
4	01/05/1998	66	Lost to follow up	Lost to follow up	Nos	WLE	1	1	Nx	Mx
5	02/04/2004	57	ALIVE	20.2	Cis	WLE	Cis	Tis	Nx	0
6	01/06/1997	65	ALIVE	101.3	Nos	WLE	3	1	1	0
7	23/05/2000	43	ALIVE	66.9	Nos	Glansectomy	1	2	1	0
8	01/05/2001	81	DEAD	48.1	Nos	Total Penectomy	3	Tx	0	1
9	10/10/2002	48	ALIVE	37.9	Nos	Total Penectomy	1	2	0	0
10	30/11/1992	43	DEAD	36.0	Nos	Partial Penectomy	3	2	1	0
11	23/09/1994	73	Lost to follow up	Lost to follow up	Nos	Total Penectomy	3	3	Nx	Mx
12	20/04/1988	60	Lost to follow up	Lost to follow up	Nos	Partial Penectomy	2	2	Nx	Mx
13	22/07/1997	56	DEAD	15.8	Nos	WLE	3	2	3	1
14	14/09/1999	69	ALIVE	62.8	Cis	WLE	Cis	Tis	0	0
15	18/06/1999	68	ALIVE	80.7	Nos	WLE	2	1	0	0
16	14/08/2003	73	ALIVE	28.7	Mixed	Glansectomy	1	2	0	0
17	01/05/1993	28	ALIVE	156.2	Cis	WLE	Cis	Tis	0	0
18	20/06/2003	50	ALIVE	35.7	Nos	Glansectomy	1	1	0	0
19	01/07/2003	66	ALIVE	31.7	Nos	WLE	2	2	2	1
20	01/04/2001	34	ALIVE	62.1	Nos	Glansectomy	2	1	0	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
21	29/04/2002	41	ALIVE	42.3	Nos	Partial Penectomy	3	3	0	0
22	01/08/1995	71	DEAD	13.9	Nos	Partial Penectomy	2	1	3	0
23	05/07/1995	80	DEAD	14.9	Nos	WLE	2	1	Nx	Mx
24	09/08/1990	75	Lost to follow up	Lost to follow up	Papillary	WLE	1	1	Nx	Mx
25	14/11/1988	73	Lost to follow up	Lost to follow up	Nos	Total Penectomy	1	2	Nx	Mx
26	12/12/1997	71	ALIVE	97.7	Verrucous	Partial Penectomy	1	1	0	0
27	02/12/1988	65	Lost to follow up	Lost to follow up	Nos	WLE	1	2	Nx	Mx
28	09/10/2000	47	ALIVE	64.2	Nos	Total Penectomy	1	3	0	0
29	05/01/2000	64	ALIVE	67.6	Basaloid	WLE	2	1	0	0
30	18/08/1998	55	Lost to follow up	Lost to follow up	Nos	WLE	2	2	Nx	Mx
31	03/12/2002	50	ALIVE	40.1	Basaloid	Glansectomy	3	1	0	0
32	19/06/2001	66	ALIVE	55.7	Nos	Total Penectomy	3	2	0	0
33	16/10/2003	75	ALIVE	25.5	Verrucous	Glansectomy	1	1	0	0
34	15/01/2003	63	ALIVE	26.1	Basaloid	Glansectomy	3	1	0	0
35	01/07/2003	57	ALIVE	31.5	Mixed	Glansectomy	1	1	0	0
36	20/11/2003	69	DEAD	9.9	Mixed	Partial Penectomy	3	2	3	0
37	15/01/2004	40	ALIVE	1.8	Cis	WLE	Cis	Tis	Nx	0
38	01/07/2004	57	ALIVE	18.4	Cis	WLE	Cis	Tis	Nx	0
39	11/07/1994	78	Lost to follow up	Lost to follow up	Nos	Partial Penectomy	2	1	Nx	Mx
40	26/10/1994	52	ALIVE	138.2	Nos	WLE	2	1	0	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
41	17/11/1993	64	ALIVE	48.1	Nos	Partial Penectomy	2	2	2	1
42	31/03/2004	43	ALIVE	22.7	Papillary	Glansectomy	1	2	0	0
43	27/02/2004	65	ALIVE	23.0	Nos	Glansectomy	2	1	Nx	0
44	10/06/2004	57	ALIVE	17.7	Nos	WLE	2	1	Nx	0
45	01/02/2000	58	DEAD	9.1	Nos	Total Penectomy	2	2	Nx	1
46	20/06/2003	62	DEAD	31.6	Papillary	Glansectomy	2	1	0	0
47	14/08/2002	69	ALIVE	36.3	Nos	Urethrectomy	1	Tx	0	0
48	01/04/1999	63	ALIVE	79.7	Nos	Partial Penectomy	3	2	0	0
49	01/08/2001	56	ALIVE	57.1	Basaloid	WLE	3	1	1	0
50	01/04/2002	39	ALIVE	50.1	Nos	Glansectomy	3	1	0	0
51	01/09/2000	47	ALIVE	65.6	Papillary	Urethrectomy	3	2	0	0
52	25/03/2002	70	ALIVE	47.4	Papillary	Total Penectomy	3	2	2	1
53	22/10/2001	69	ALIVE	44.8	Nos	Partial Penectomy	2	1	0	0
54	01/03/2000	55	ALIVE	1.3	Nos	Glansectomy	2	2	Nx	0
55	07/03/2000	56	DEAD	11.8	Nos	Total Penectomy	2	Tx	2	Mx
56	16/09/1993	39	DEAD	137.5	Mixed	Glansectomy	2	2	1	1
57	18/01/2001	35	DEAD	20.2	Nos	Partial Penectomy	2	2	3	1
58	21/04/2005	75	DEAD	1.1	Papillary	Glansectomy	3	2	3	Mx
59	01/05/2002	39	ALIVE	45.5	Papillary	Glansectomy	1	2	0	0
60	05/06/1991	33	Lost to follow up	Lost to follow up	Nos	WLE	1	1	Nx	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
61	12/05/1988	81	DEAD	3.6	Nos	Penectomy	2	2	Nx	Mx
62	06/05/2004	72	ALIVE	25.1	Nos	Glansectomy	2	1	0	0
63	19/12/1991	54	DEAD	5.0	Nos	Total Penectomy	1	2	3	0
64	28/08/2002	77	DEAD	37.0	Nos	Total Penectomy	3	3	2	0
65	23/01/2001	35	ALIVE	48.5	Nos	Glansectomy	2	1	0	0
66	27/01/2005	71	ALIVE	12.4	Nos	Glansectomy	3	1	Nx	0
67	10/10/2003	57	ALIVE	27.3	Mixed	Glansectomy	1	1	0	0
68	19/02/2004	52	ALIVE	27.7	Cis	WLE	Cis	Tis	0	0
69	16/05/2000	57	ALIVE	64.9	Verrucous	Partial Penectomy	1	2	0	0
70	01/06/1992	54	Lost to follow up	Lost to follow up	Nos	WLE	2	1	Nx	Mx
71	22/10/1996	56	ALIVE	107.9	Nos	WLE	3	1	0	Mx
72	19/02/1990	73	ALIVE	139.7	Basaloid	WLE	3	1	0	Mx
73	01/02/1992	52	ALIVE	162.4	Nos	WLE	2	1	0	Mx
74	30/07/2004	63	DEAD	3.0	Nos	Partial Penectomy	3	2	2	1
75	20/01/2005	60	ALIVE	15.4	Nos	Glansectomy	2	2	0	0
76	18/07/2000	56	ALIVE	64.4	Cis	WLE	Cis	Tis	0	0
77	07/08/2002	45	ALIVE	42.9	Nos	Partial Penectomy	3	1	2	0
78	10/12/2004	72	ALIVE	13.7	Nos	Partial Penectomy	1	2	0	0
79	01/07/2003	55	ALIVE	28.1	Nos	WLE	2	1	0	0
80	02/12/2004	63	ALIVE	18.0	Nos	WLE	2	1	Nx	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
81	10/12/2004	74	ALIVE	17.8	Mixed	Partial Penectomy	3	1	0	0
82	01/06/2003	54	ALIVE	33.2	Basaloid	Glansectomy	3	2	0	0
83	13/10/1989	74	Lost to follow up	Lost to follow up	Cis	WLE	Cis	Tis	Nx	0
84	03/03/2004	79	ALIVE	23.0	Nos	Glansectomy	3	1	0	0
85	15/04/2004	60	ALIVE	22.2	Nos	Glansectomy	2	2	1	0
86	16/02/2000	47	ALIVE	67.4	Nos	Total Penectomy	2	3	0	0
87	22/12/1994	57	ALIVE	133.6	Nos	Partial Penectomy	1	1	0	0
88	15/05/2000	45	DEAD	32.2	Nos	Urethrectomy	3	1	0	1
89	13/01/2005	38	ALIVE	12.9	Verrucous	WLE	1	1	Nx	0
90	15/07/2005	80	ALIVE	7.6	Nos	WLE	2	1	Nx	0
91	01/06/2005	79	ALIVE	7.5	Mixed	WLE	3	1	0	0
92	08/03/2005	62	ALIVE	15.0	Nos	WLE	3	1	3	1
93	17/08/2005	53	ALIVE	6.0	Mixed	Partial Glansectomy	3	1	0	0
94	15/09/2005	59	ALIVE	2.3	Cis	WLE	Cis	Tis	Nx	0
95	01/09/2003	40	ALIVE	33.3	Papillary	Partial Penectomy	2	1	0	0
96	15/07/2004	39	ALIVE	21.8	Papillary	Glansectomy	2	2	0	0
97	14/02/2004	79	DEAD	19.0	Nos	Partial Penectomy	2	2	2	1
98	30/11/1992	UKNOWN	Lost to follow up	Lost to follow up	Nos	WLE	2	1	Nx	Mx
99	01/06/2005	61	ALIVE	8.3	Papillary	Glansectomy	1	2	Nx	0
100	01/09/2005	63	DEAD	17.0	Mixed	Partial Penectomy	2	3	Nx	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
101	09/03/2005	55	ALIVE	13.9	Nos	Glansectomy	3	2	2	0
102	04/03/2005	59	ALIVE	13.0	Nos	Glansectomy	3	2	2	0
103	01/06/2003	67	DEAD	21.8	Nos	Partial Penectomy	2	2	1	0
104	15/02/2005	63	ALIVE	13.9	Papillary	Glansectomy	2	1	Nx	0
105	28/01/2004	66	ALIVE	20.5	Papillary	Total Penectomy	2	2	0	0
106	23/06/2005	42	ALIVE	9.6	Nos	Total Penectomy	2	3	0	0
107	01/08/2003	75	ALIVE	30.2	Papillary	Partial Penectomy	3	3	1	0
108	22/07/2005	82	ALIVE	6.5	Nos	Total Penectomy	3	3	2	1
109	02/05/2006	63	ALIVE	1.1	Mixed	Total Penectomy	2	4	0	0
110	09/01/2006	70	ALIVE	2.8	Papillary	Glansectomy	2	2	0	0
111	01/12/2001	46	ALIVE	48.7	Nos	Glansectomy	2	2	0	0
112	08/12/2005	69	ALIVE	2.0	Nos	Glansectomy	2	1	Nx	0
113	23/10/2002	75	DEAD	15.0	Papillary	Partial Penectomy	3	1	2	1
114	13/01/2006	36	ALIVE	0.9	Cis	Glansectomy	Cis	Tis	Nx	0
115	01/02/2006	42	ALIVE	2.6	Nos	Glansectomy	2	2	1	0
116	24/11/2005	71	ALIVE	3.0	Nos	Glansectomy	2	1	Nx	0
117	24/11/2005	55	ALIVE	6.4	Nos	Total Penectomy	3	3	0	1
118	04/05/2005	58	ALIVE	10.0	Nos	Glansectomy	3	1	0	0
119	26/01/2006	28	ALIVE	3.8	Nos	WLE	2	1	Nx	0
120	28/12/2005	60	ALIVE	4.2	Nos	Glansectomy	2	1	Nx	0

Case ID	Date Diagnosis	Age	Dead or Alive	Survival (months)	Subtype	Operation	Grade	T Stage	N Stage	M Stage
121	02/03/2005	61	DEAD	24.3	Nos	Glansectomy	2	2	3	0
122	20/01/2006	55	ALIVE	3.5	Nos	Glansectomy	3	3	0	0
123	02/12/2004	87	DEAD (NCR)	3.3	Nos	Partial penectomy	1	2	0	0
124	18/01/2006	70	ALIVE	4.4	Nos	Glansectomy	3	1	1	0
125	09/11/2005	77	ALIVE	6.2	Nos	Partial Penectomy	3	2	0	0
126	30/08/2002	63	ALIVE	41.8	Papillary	Partial Penectomy	3	2	0	0
127	27/10/2005	79	ALIVE	3.2	Mixed	Glansectomy	3	2	Nx	0
128	27/04/2006	34	ALIVE	1.5	Nos	Glansectomy	2	2	0	0
129	27/03/2006	70	ALIVE	2.5	Basaloid	Glansectomy	3	2	Nx	0
130	19/05/2006	55	ALIVE	0.8	Nos	Partial penectomy	1	2	Nx	0
131	22/03/2006	59	ALIVE	2.7	Warty	WLE	2	2	Nx	0
132	01/04/2003	72	ALIVE	36.1	Papillary	Partial Penectomy	3	1	2	0
133	24/05/2006	59	ALIVE	6.4	Nos	Glansectomy	3	2	1	0
134	02/06/2006	64	ALIVE	5.9	Nos	Glansectomy	2	2	0	0
135	19/06/2006	72	ALIVE	4.4	Cis	Circumcision	Cis	Tis	0	0
136	25/07/2006	47	ALIVE	3.4	Nos	Glansectomy	3	2	1	0
137	18/09/2006	65	ALIVE	2.2	Nos	Hemi-glansectomy	3	1	Nx	0
138	02/10/2006	76	ALIVE	3.9	Warty	WLE	2	1	Nx	0
139	26/09/2006	38	ALIVE	3.3	Papillary	Glansectomy	2	1	0	0
140	30/09/2006	83	DEAD	2.5	Nos	Total Penectomy	3	4	2	1
141	15/02/2006	85	DEAD	4.9	Nos	Partial Penectomy	3	2	2	0

APPENDIX B

Summary of protein expression profile analyses

Case ID	Mcm2			Ki-67			G95			Mcm2/Ki67	G95/Ki67	Ki67-G95	Mcm2-Ki67	Ploidy
	Positive	Negative	LI (%)	Positive	Negative	LI (%)	Positive	Negative	LI (%)					
1	1004	113	89.9	988	188	84.0	287	900	24.2	1.07	0.29	59.8	5.9	Diploid
2	372	113	76.7	508	234	68.5	79	402	16.4	1.12	0.24	52.0	8.2	Unknown
3	2063	84	96.1	1322	419	75.9	422	1331	24.1	1.27	0.32	51.9	20.2	Diploid
4	626	320	66.2	510	344	59.7	115	665	14.7	1.11	0.25	45.0	6.5	Diploid
5	2044	108	95.0	2029	140	93.5	385	1042	27.0	1.02	0.29	66.6	1.4	Diploid
6	1515	213	87.7	1207	208	85.3	304	1214	20.0	1.03	0.23	65.3	2.4	Diploid
7	543	414	56.7	545	400	57.7	167	591	22.0	0.98	0.38	35.6	-0.9	Diploid
8	657	136	82.8	690	99	87.5	180	373	32.5	0.95	0.37	54.9	-4.6	Aneuploid
9	1043	207	83.4	891	148	85.8	214	907	19.1	0.97	0.22	66.7	-2.3	Diploid
10	247	417	37.2	943	902	51.1	280	928	23.2	0.73	0.45	27.9	-13.9	Aneuploid
11	345	275	55.6	215	513	29.5	108	499	17.8	1.88	0.60	11.7	26.1	Aneuploid
12	785	116	87.1	785	203	79.4	222	808	21.6	1.10	0.27	57.9	7.7	Unknown
13	839	122	87.3	1293	218	85.6	511	1475	25.7	1.02	0.30	59.8	1.7	Aneuploid
14	1122	106	91.4	1135	225	83.5	208	906	18.7	1.09	0.22	64.8	7.9	Diploid
15	722	261	73.4	572	297	65.8	162	665	19.6	1.12	0.30	46.2	7.6	Diploid
16	942	195	82.8	581	483	54.6	190	683	21.8	1.52	0.40	32.8	28.2	Diploid
17	654	147	81.6	581	268	68.4	251	513	32.9	1.19	0.48	35.6	13.2	Unknown
18	1064	296	78.2	774	570	57.6	172	799	17.7	1.36	0.31	39.9	20.6	Diploid
19	2111	64	97.1	3037	130	95.9	488	1662	22.7	1.01	0.24	73.2	1.2	Aneuploid
20	1486	386	79.4	1570	317	83.2	328	1536	17.6	0.95	0.21	65.6	-3.8	Aneuploid
21	832	166	83.4	791	184	81.1	254	748	25.3	1.03	0.31	55.8	2.2	Aneuploid
22	445	327	57.6	728	394	64.9	234	872	21.2	0.89	0.33	43.7	-7.2	Aneuploid
23	875	146	85.7	708	342	67.4	127	748	14.5	1.27	0.22	52.9	18.3	Unknown
24	342	290	54.1	164	510	24.3	31	693	4.3	2.22	0.18	20.1	29.8	Diploid
25	229	247	48.1	164	339	32.6	23	465	4.7	1.48	0.14	27.9	15.5	Unknown
26	176	293	37.5	135	320	29.7	67	714	8.6	1.26	0.29	21.1	7.9	Diploid
27	446	372	54.5	354	650	35.3	87	880	9.0	1.55	0.26	26.3	19.3	Unknown
28	298	390	43.3	266	446	37.4	78	634	11.0	1.16	0.29	26.4	6.0	Diploid
29	1973	252	88.7	1483	393	79.1	479	1263	27.5	1.12	0.35	51.6	9.6	Diploid
30	781	425	64.8	811	1226	39.8	153	2987	4.9	1.63	0.12	34.9	24.9	Unknown

Case ID	Mcm2			Ki-67			G95			Mcm2/Ki67	G95/Ki67	Ki67-G95	Mcm2-Ki67	Ploidy
	Positive	Negative	LI (%)	Positive	Negative	LI (%)	Positive	Negative	LI (%)					
31	2383	12	99.5	2366	97	96.1	536	1608	25.0	1.04	0.26	71.1	3.4	Aneuploid
32	759	273	73.5	778	554	58.4	256	716	26.3	1.26	0.45	32.1	15.1	Diploid
33	258	509	33.6	210	573	26.8	35	710	4.7	1.25	0.18	22.1	6.8	Diploid
34	2942	111	96.4	2450	789	75.6	869	2186	28.4	1.27	0.38	47.2	20.7	Diploid
35	148	137	51.9	1304	1490	46.7	83	822	9.2	1.11	0.20	37.5	5.3	Diploid
36	2525	50	98.1	2577	124	95.4	994	1663	37.4	1.03	0.39	58.0	2.6	Aneuploid
37	916	28	97.0	668	71	90.4	233	1089	17.6	1.07	0.19	72.8	6.6	Diploid
38	1036	67	93.9	769	375	67.2	170	986	14.7	1.40	0.22	52.5	26.7	Unknown
39	1035	216	82.7	1020	240	81.0	360	1209	22.9	1.02	0.28	58.0	1.8	Diploid
40	546	94	85.3	404	157	72.0	66	659	9.1	1.18	0.13	62.9	13.3	Diploid
41	828	410	66.9	653	475	57.9	284	690	29.2	1.16	0.50	28.7	9.0	Unknown
42	1299	325	80.0	1082	297	78.5	287	873	24.7	1.02	0.32	53.7	1.5	Diploid
43	715	98	87.9	731	132	84.7	171	554	23.6	1.04	0.28	61.1	3.2	Diploid
44	2339	87	96.4	1869	412	81.9	570	2374	19.4	1.18	0.24	62.6	14.5	Aneuploid
45	862	153	84.9	992	200	83.2	213	666	24.2	1.02	0.29	59.0	1.7	Aneuploid
46	1054	192	84.6	1057	374	73.9	222	641	25.7	1.15	0.35	48.1	10.7	Aneuploid
47	541	179	75.1	400	187	68.1	111	394	22.0	1.10	0.32	46.2	7.0	Diploid
48	2030	29	98.6	1826	28	98.5	570	1425	28.6	1.00	0.29	69.9	0.1	Aneuploid
49	3141	9	99.7	2246	131	94.5	1041	1762	37.1	1.06	0.39	57.4	5.2	Aneuploid
50	167	22	88.4	315	75	80.8	68	298	18.6	1.09	0.23	62.2	7.6	Aneuploid
51	1923	23	98.8	1522	328	82.3	397	1567	20.2	1.20	0.25	62.1	16.5	Unknown
52	2893	57	98.1	2947	40	98.7	951	1700	35.9	0.99	0.36	62.8	-0.6	Aneuploid
53	1245	185	87.1	1147	247	82.3	411	1071	27.7	1.06	0.34	54.5	4.8	Aneuploid
54	3069	143	95.5	3242	211	93.9	824	2156	27.7	1.02	0.29	66.2	1.7	Diploid
55	1965	107	94.8	1771	207	89.5	591	1490	28.4	1.06	0.32	61.1	5.3	Aneuploid
56	819	730	52.9	1020	375	73.1	285	735	27.9	0.72	0.38	45.2	-20.2	Aneuploid
57	586	502	53.9	511	676	43.0	139	1315	9.6	1.25	0.22	33.5	10.8	Aneuploid
58	1877	121	93.9	1735	263	86.8	355	1790	16.6	1.08	0.19	70.3	7.1	Unknown
59	535	1873	22.2	396	1842	17.7	159	1678	8.7	1.26	0.49	9.0	4.5	Diploid
60	485	1062	31.4	296	1082	21.5	221	1114	16.6	1.46	0.77	4.9	9.9	Unknown

Case ID	Mcm2			Ki-67			G95			Mcm2/Ki67	G95/Ki67	Ki67-G95	Mcm2-Ki67	Ploidy
	Positive	Negative	LI (%)	Positive	Negative	LI (%)	Positive	Negative	LI (%)					
61	588	120	83.1	430	89	82.9	174	596	22.6	1.00	0.27	60.3	0.2	Diploid
62	987	531	65.0	1130	511	68.9	215	1477	12.7	0.94	0.18	56.2	-3.8	Diploid
63	800	512	61.0	725	578	55.6	334	1164	22.3	1.10	0.40	33.3	5.3	Aneuploid
64	1782	382	82.3	1583	804	66.3	445	1815	19.7	1.24	0.30	46.6	16.0	Aneuploid
65	897	900	49.9	562	1602	26.0	108	1024	9.5	1.92	0.37	16.4	23.9	Diploid
66	461	437	51.3	278	268	50.9	178	838	17.5	1.01	0.34	33.4	0.4	Aneuploid
67	308	333	48.0	441	515	46.1	50	660	7.0	1.04	0.15	39.1	1.9	Diploid
68	202	66	75.4	124	119	51.0	28	222	11.2	1.48	0.22	39.8	24.3	Unknown
69	244	336	42.1	215	391	35.5	78	578	11.9	1.19	0.34	23.6	6.6	Diploid
70	1145	326	77.8	586	894	39.6	192	1211	13.7	1.97	0.35	25.9	38.2	Diploid
71	2056	378	84.5	1599	779	67.2	410	1636	20.0	1.26	0.30	47.2	17.2	Unknown
72	3261	80	97.6	1904	1053	64.4	607	3322	15.4	1.52	0.24	48.9	33.2	Aneuploid
73	841	1284	39.6	825	1210	40.5	180	660	21.4	0.98	0.53	19.1	-1.0	Diploid
74	472	165	74.1	516	324	61.4	84	769	9.8	1.21	0.16	51.6	12.7	Aneuploid
75	291	608	32.4	343	903	27.5	69	960	6.7	1.18	0.24	20.8	4.8	Unknown
76	715	63	91.9	826	445	65.0	121	694	14.8	1.41	0.23	50.1	26.9	Aneuploid
77	598	129	82.3	484	212	69.5	89	372	19.3	1.18	0.28	50.2	12.7	Diploid
78	376	265	58.7	427	355	54.6	106	539	16.4	1.07	0.30	38.2	4.1	Diploid
79	2183	120	94.8	2093	204	91.1	241	1663	12.7	1.04	0.14	78.5	3.7	Aneuploid
80	1768	76	95.9	1666	55	96.8	434	1232	26.1	0.99	0.27	70.8	-0.9	Diploid
81	891	263	77.2	767	441	63.5	150	757	16.5	1.22	0.26	47.0	13.7	Aneuploid
82	1746	148	92.2	1002	198	83.5	225	835	21.2	1.10	0.25	62.3	8.7	Aneuploid
83	1104	80	93.2	1159	155	88.2	319	1028	23.7	1.06	0.27	64.5	5.0	Aneuploid
84	2016	248	89.0	1994	120	94.3	430	1617	21.0	0.94	0.22	73.3	-5.3	Aneuploid
85	1831	1279	58.9	1453	1424	50.5	219	3005	6.8	1.17	0.13	43.7	8.4	Diploid
86	525	447	54.0	817	539	60.3	214	921	18.9	0.90	0.31	41.4	-6.2	Aneuploid
87	229	510	31.0	305	657	31.7	74	672	9.9	0.98	0.31	21.8	-0.7	Unknown
88	1035	680	60.3	1021	1010	50.3	290	1485	16.3	1.20	0.33	33.9	10.1	Aneuploid
89	280	1117	20.0	354	1133	23.8	43	1628	2.6	0.84	0.11	21.2	-3.8	Aneuploid
90	979	378	72.1	962	430	69.1	248	1137	17.9	1.04	0.26	51.2	3.0	Aneuploid

Case ID	Mcm2			Ki-67			G95			Mcm2/Ki67	G95/Ki67	Ki67-G95	Mcm2-Ki67	Ploidy
	Positive	Negative	LI (%)	Positive	Negative	LI (%)	Positive	Negative	LI (%)					
91	2970	50	98.3	2970	243	92.4	353	2970	10.6	1.06	0.11	81.8	5.9	Aneuploid
92	1848	117	94.0	1243	845	59.5	455	1693	21.2	1.58	0.36	38.3	34.5	Unknown
93	1957	161	92.4	4312	452	90.5	721	4381	14.1	1.02	0.16	76.4	1.9	Aneuploid
94	817	233	77.8	771	485	61.4	85	832	9.3	1.27	0.15	52.1	16.4	Diploid
95	2114	110	95.1	2619	208	92.6	709	1816	28.1	1.03	0.30	64.6	2.4	Aneuploid
96	1527	356	81.1	1427	661	68.3	418	1600	20.7	1.19	0.30	47.6	12.8	Diploid
97	457	236	65.9	737	440	62.6	191	938	16.9	1.05	0.27	45.7	3.3	Diploid
98	633	217	74.5	837	617	57.6	133	1515	8.1	1.29	0.14	49.5	16.9	Aneuploid
99	990	718	58.0	493	1288	27.7	79	1638	4.6	2.09	0.17	23.1	30.3	Aneuploid
100	1406	237	85.6	1620	611	72.6	283	1497	15.9	1.18	0.22	56.7	13.0	Aneuploid
101	610	142	81.1	575	405	58.7	298	521	36.4	1.38	0.62	22.3	22.4	Aneuploid
102	575	112	83.7	471	168	73.7	205	489	29.5	1.14	0.40	44.2	10.0	Aneuploid
103	1618	96	94.4	1433	266	84.3	552	1305	29.7	1.12	0.35	54.6	10.1	Aneuploid
104	261	200	56.6	167	147	53.2	48	299	13.8	1.06	0.26	39.4	3.4	Aneuploid
105	1892	342	84.7	1897	492	79.4	570	1221	31.8	1.07	0.40	47.6	5.3	Diploid
106	741	251	74.7	667	147	81.9	212	620	25.5	0.91	0.31	56.5	-7.2	Aneuploid
107	1317	473	73.6	1014	394	72.0	380	1108	25.5	1.02	0.35	46.5	1.6	Aneuploid
108	932	623	59.9	625	557	52.9	224	1339	14.3	1.13	0.27	38.5	7.1	Unknown
109	520	121	81.1	422	211	66.7	138	535	20.5	1.22	0.31	46.2	14.4	Aneuploid
110	752	378	66.5	639	504	55.9	239	790	23.2	1.19	0.42	32.7	10.6	Aneuploid
111	1101	241	82.0	829	555	59.9	318	1000	24.1	1.37	0.40	35.8	22.1	Unknown
112	1834	18	99.0	1795	35	98.1	641	1232	34.2	1.01	0.35	63.9	0.9	Aneuploid
113	1472	75	95.2	1365	215	86.4	436	1040	29.5	1.10	0.34	56.9	8.8	Aneuploid
114	2400	12	99.5	2400	75	97.0	496	2006	19.8	1.03	0.20	77.1	2.5	Aneuploid
115	567	462	55.1	693	340	67.1	162	795	16.9	0.82	0.25	50.2	-12.0	Aneuploid
116	519	641	44.7	467	684	40.6	78	746	9.5	1.10	0.23	31.1	4.2	Aneuploid
117	605	96	86.3	741	190	79.6	234	443	34.6	1.08	0.43	45.0	6.7	Aneuploid
118	873	434	66.8	463	655	41.4	311	1315	19.1	1.61	0.46	22.3	25.4	Aneuploid
119	1433	284	83.5	1278	485	72.5	405	1110	26.7	1.15	0.37	45.8	11.0	Aneuploid
120	792	396	66.7	488	475	50.7	107	978	9.9	1.32	0.19	40.8	16.0	Aneuploid

Case ID	Mcm2			Ki-67			G95			Mcm2/Ki67	G95/Ki67	Ki67-G95	Mcm2-Ki67	Ploidy
	Positive	Negative	LI (%)	Positive	Negative	LI (%)	Positive	Negative	LI (%)					
121	404	236	63.1	265	254	51.1	116	470	19.8	1.24	0.39	31.3	12.1	Aneuploid
122	1119	232	82.8	1126	360	75.8	407	850	32.4	1.09	0.43	43.4	7.1	Aneuploid
123	3595	880	80.3	563	1063	34.6	154	1321	10.4	2.32	0.30	24.2	45.7	Aneuploid
124	1111	77	93.5	1012	156	86.6	334	834	28.6	1.08	0.33	58.0	6.9	Aneuploid
125	988	56	94.6	957	96	90.9	499	570	46.7	1.04	0.51	44.2	3.8	Aneuploid
126	1243	42	96.7	1199	190	86.3	338	1049	24.4	1.12	0.28	62.0	10.4	Aneuploid
127	1290	25	98.1	1458	168	89.7	531	1330	28.5	1.09	0.32	61.1	8.4	Aneuploid
128	658	176	78.9	552	246	69.2	198	657	23.2	1.14	0.33	46.0	9.7	Diploid
129	768	89	89.6	552	363	60.3	66	699	8.6	1.49	0.14	51.7	29.3	Diploid
130	507	340	59.9	279	371	42.9	86	614	12.3	1.39	0.29	30.6	16.9	Diploid
131	1085	243	81.7	781	649	54.6	251	1019	19.8	1.50	0.36	34.9	27.1	Aneuploid
132	1350	99	93.2	1350	140	90.6	509	723	41.3	1.03	0.46	49.3	2.6	Unknown
133	2158	258	89.3	1869	423	81.5	251	2144	10.5	1.10	0.13	71.1	7.8	Aneuploid
134	310	727	29.9	310	923	25.1	23	1376	1.6	1.19	0.07	23.5	4.8	Aneuploid
135	2176	193	91.9	1896	479	79.8	360	2001	15.3	1.15	0.19	64.6	12.0	Aneuploid
136	1541	470	76.6	775	1358	36.3	700	1989	26.0	2.11	0.72	10.3	40.3	Aneuploid
137	1619	139	92.1	1565	537	74.6	238	1529	13.5	1.24	0.18	61.1	17.5	Aneuploid
138	1065	1028	50.9	873	1080	44.7	75	1700	4.3	1.14	0.10	40.4	6.2	Aneuploid
139	1033	78	93.0	1188	364	76.6	93	1356	6.4	1.21	0.08	70.1	16.4	Aneuploid
140	1798	440	80.3	468	972	32.5	181	1351	11.8	2.47	0.36	20.7	47.8	Aneuploid
141	815	198	87.9	831	232	84.7	260	643	23.6	1.04	0.28	61.1	3.2	Diploid

APPENDIX C

Supportive documents

(Form to be on headed paper)
CONFIDENTIAL

Centre Number: UCLH Project ID number:
Patient Identification Number for this study: Form version: 1.1

CONSENT FORM

Prognostic Histopathological Features and Markers in Penile Cancer

Principal investigator :

The Institute of Urology and Nephrology, UCL, Middlesex Hospital, 48, Riding House Street, London W1W 7EY

Please initial box

1. I confirm that I have read and understood the information sheet dated January 2004 (version 1.1) for the above study and have had the opportunity to ask questions. ☐
2. I confirm that I have had sufficient time to consider whether or not want to be included in the study ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
4. I understand that sections of any of my medical notes may be looked at by responsible individuals from (company name) or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
5. I agree to take part in the above study. ☐

Continued on next page/

1 form for Patient;
1 to be kept as part of the study documentation,
1 to be kept with hospital notes

(Form to be on headed paper)

Centre Number: UCLH Project ID number:
Patient Identification Number for this study: Form version: 1.1

CONSENT FORM

Title of project: Prognostic Pathological Characteristics and Markers in Penile Cancer

Principal Investigator : Mr S. Minhas

Name of patient	Date	Signature

Name of Person taking consent (if different from researcher)	Date	Signature

Dr Oliver Kayes Research fellow

Name of the researcher to be contacted if there are any problems

Comments or concerns during the study

If you have any comments or concerns you may discuss these with the investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL hospitals. Please quote the UCLH project number at the top this consent form.

1 form for Patient;
1 to be kept as part of the study documentation,
1 to be kept with hospital notes

(Form to be on headed paper)
CONFIDENTIAL

Centre Number: UCLH Project ID number:
Patient Identification Number for this study: Form version: 1.1
Date: Jan 2004

PATIENT INFORMATION SHEET

Prognostic Histopathological Features and Markers in Penile Cancer

(A study on penile cancer tissue removed at operation)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Penile cancer is a relatively rare disease, in order to understand more about it we need to analyse as many samples as possible. The aim is to be able to predict the behaviour of the disease from looking at specimens under the microscope, this way we will be able to offer the right treatment to the right people.

Why have I been chosen?

Everyone with disease of the penis; which may be penis cancer, will be asked to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What is involved in the study?

If, as part of the treatment of your disease, you have an operation, we will want to study the tissue removed.

It is important to realise that taking part in this study will not alter how you are treated in any way.

The tests, treatments or operations and follow up visits to the hospital afterwards will be the same regardless of whether you take part or not.
We will however collect data on who you are, the type of disease you have and the long term outcome of your disease. This will include collection of blood and urine samples.

What are the possible benefits of taking part?

There is no intended clinical benefit to you by taking part in this study, however the information we get from this study may help us to treat future patients with penile cancer better.

The information held about you

The study will involve holding information on your case, which will include your clinical details and the pathology results from the tissue analysed. UCLH will act as "data controller", that is to say will collect, store, handle and process the data. The safety and security of the data will be the responsibility of the principal investigator, Mr S. Minhas, Senior Lecturer in Urology. Access to the data will be restricted to those directly involved in the study of penile cancer as approved by Mr S. Minhas.

All information that is collected about you during the course of the study will be kept strictly confidential.

Any information about you that leaves the hospital will have your name and address, date of birth and all identifiable information (including patient/hospital/NHS number) removed so that you cannot be recognised from it. The exception to this being that during the course of your treatment your GP will be informed as per normal practice of your progress.

Tissue storage

The sample donated will be considered a gift, and may be retained for future research. Any new research will be reviewed by a research ethics committee, but consent for future studies will only be required if the committee considers that the study is likely to substantially affect your interests.

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Results of the study

You will receive the basic results of your tests (where the tissue is looked at under the microscope) should you wish to know them, in the same way that you normally would. The results from additional specialist tests on the tissue will not routinely be made known to you, unless there is clear evidence demonstrating its significance.
The results will ultimately be published in a medical journal, and this is likely to be within the next couple of years (though of course you will not be identified in any report or publication).

Research funding

This study will be funded by a charitable organisation, called the St. Peters Urology Fund, and this fund will pay for including you in this study. No payment will be made to the doctors conducting the research for including you in this study.

Withdrawal from the project

Your participation in the trial is entirely voluntary. You are free to decline to enter or to withdraw from the study any time without having to give a reason. If you choose not to enter the trial, or to withdraw once entered, this will in no way affect your future medical care. All information regarding your medical records will be treated as strictly confidential and will only be used for medical purposes. Your medical records may be inspected by competent authorities and properly authorised persons, but if any information is released this will be done in a coded form so that confidentiality is strictly maintained. Participation in this study will in no way affect your legal rights.

Who has reviewed the study?

This study has been reviewed by the Joint UCL/UCLH Committees on the ethics of human research which are administered by the UCLH Research & Development Directorate.

Contact for further information

If you have any further queries please contact Mr O J Kayes, Research fellow at the Andrology Department, The Institute of Urology and Nephrology, UCL, Middlesex Hospital, 48, Riding House Street, London W1W

Finally,

Thank-you for taking part in this study!

You will be given a copy of this information sheet and a signed consent form to keep.

PENILE CANCER PROFORMA

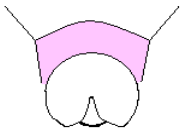
DATE SEEN BY

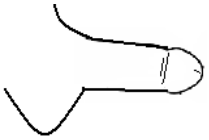
CONSULTANT

NAME .....
HOSPITAL NUMBER .....
DOB .....
AGE .....
ADDRESS .....
.....
.....
.....
CONTACT TELEPHONE .....

REFERRING CENTRE .....

SITE





CO-MORBIDITY:

RISK FACTORS:

BXO ☐
STD ☐
UNCIRCUMCISED ☐
SMOKER ☐
PROFESSION:

SEXUAL ACTIVITY:

CLINICAL

Inguinal lymph nodes palpable? Y/N Site?

Please attach to patient's notes. **ALL** notes to be returned to St. Peters' Office Level 2 (C/o Mr Sux Minhas)

OPERATIVE HISTORY AND HISTOLOGY **NAME:**

BIOPSY Date Procedure
Surgeon
Centre
Complications

PRIMARY EXCISION
Date Procedure
Surgeon
Centre
Complications

RECURRENCE
Date Procedure

Surgeon
Centre
Complications

LYMPH NODES

Date: Palpable Y/N

R L NUMBER: TOTAL IP STAY:
INGUINAL PELVIC
SUPERFICIAL RADICAL COMPLICATIONS: COMMENTS:
FROZEN TIC
POSITIVE NEGATIVE DRAINS:

Date: Palpable Y/N

R L NUMBER: TOTAL IP STAY:
INGUINAL PELVIC
SUPERFICIAL RADICAL COMPLICATIONS: COMMENTS:
FROZEN TIC
POSITIVE NEGATIVE DRAINS:

Date: Palpable Y/N

R L NUMBER: TOTAL IP STAY:
INGUINAL PELVIC
SUPERFICIAL RADICAL COMPLICATIONS: COMMENTS:
FROZEN TIC
POSITIVE NEGATIVE DRAINS:

HISTOLOGY **NAME:**

SPECIMEN

SUBTYPE STANDARD SCC BASALOID VERRUCIFORM PAPILLARY SARCOMATOID

GRADE 1 2 3 ▪ DIMENSIONS

STAGE X 0 1 2 3 4 ▪ DEPTH INVASION

▪ LIMITS

▪ VASCULAR INVASION Y/N
IMMUNO Y/N

NODES L R FROZEN
INGUINAL PELVIC NUMBER
SUPERFICIAL DEEP POSITIVE NEGATIVE

L R FROZEN
INGUINAL PELVIC NUMBER
SUPERFICIAL DEEP POSITIVE NEGATIVE

L R FROZEN

INGUINAL SUPERFICIAL	PELVIC DEEP	NUMBER POSITIVE	NEGATIVE

COMMENTS

IMAGING **NAME:**

MRI

DATE: PRIMARY/RECURRENCE/NODES
PGE-1 Y/N RESPONSE None/Poor/Good
SEQUENCE
T (Stage) X 0 1 2 3 4
N (Nodes) X 0 1 2 3

Comments:

DATE: PRIMARY/RECURRENCE/NODES
PGE-1 Y/N RESPONSE None/Poor/Good
SEQUENCE
T (Stage) X 0 1 2 3 4
N (Nodes) X 0 1 2 3

Comments:

DATE: PRIMARY/RECURRENCE/NODES
PGE-1 Y/N RESPONSE None/Poor/Good
SEQUENCE
T (Stage) X 0 1 2 3 4
N (Nodes) X 0 1 2 3

Comments:

Tx - unassessable
T0 - no evidence of tumour
T1 - invades subepithelial connective tissue
T2 - invades corpus spongiosum or corpus cavernosum
T3 - invades urethra or prostate
T4 - invades adjacent structures

Nx - unassessable
N0 - no lymph node involvement
N1 - single inguinal lymph node
N2 - multiple or bilateral superficial inguinal nodes
N3 - deep inguinal or pelvic nodes

PET Y/N Dates:

CT Y/N Dates:

USS/FNA Y/N Dates:

PLEASE ATTACH ALL RADIOLOGY REPORTS

SENTINEL NODE **NAME:**

Intraoperative Counts
Injection site count :
Background count :
Residual groin post SLNB :

SN1 :	Hot? :	Gamma counts/sec :		In Vivo :
	Blue? :	Strong	Weak	Ex Vivo :
Histology :	Micromets	Macromets	Extracapsular Invasion	
Notes :				

SN2 :	Hot? :	Gamma counts/sec :		In Vivo :
	Blue? :	Strong	Weak	Ex Vivo :
Histology :	Micromets	Macromets	Extracapsular Invasion	
Notes :				

SN3 :	Hot? :	Gamma counts/sec :		In Vivo :
	Blue? :	Strong	Weak	Ex Vivo :
Histology :	Micromets	Macromets	Extracapsular Invasion	
Notes :				

SN4 :	Hot? :	Gamma counts/sec :		In Vivo :
	Blue? :	Strong	Weak	Ex Vivo :
Histology :	Micromets	Macromets	Extracapsular Invasion	
Notes :				

SN5 :	Hot? :	Gamma counts/sec :	Gamma counts	In Vivo :
	Blue? :	Strong	Weak	Ex Vivo :
Histology :	Micromets	Macromets	Extracapsular Invasion	
Notes :				

OUTPATIENTS **NAME**

DATE	

OUTPATIENTS

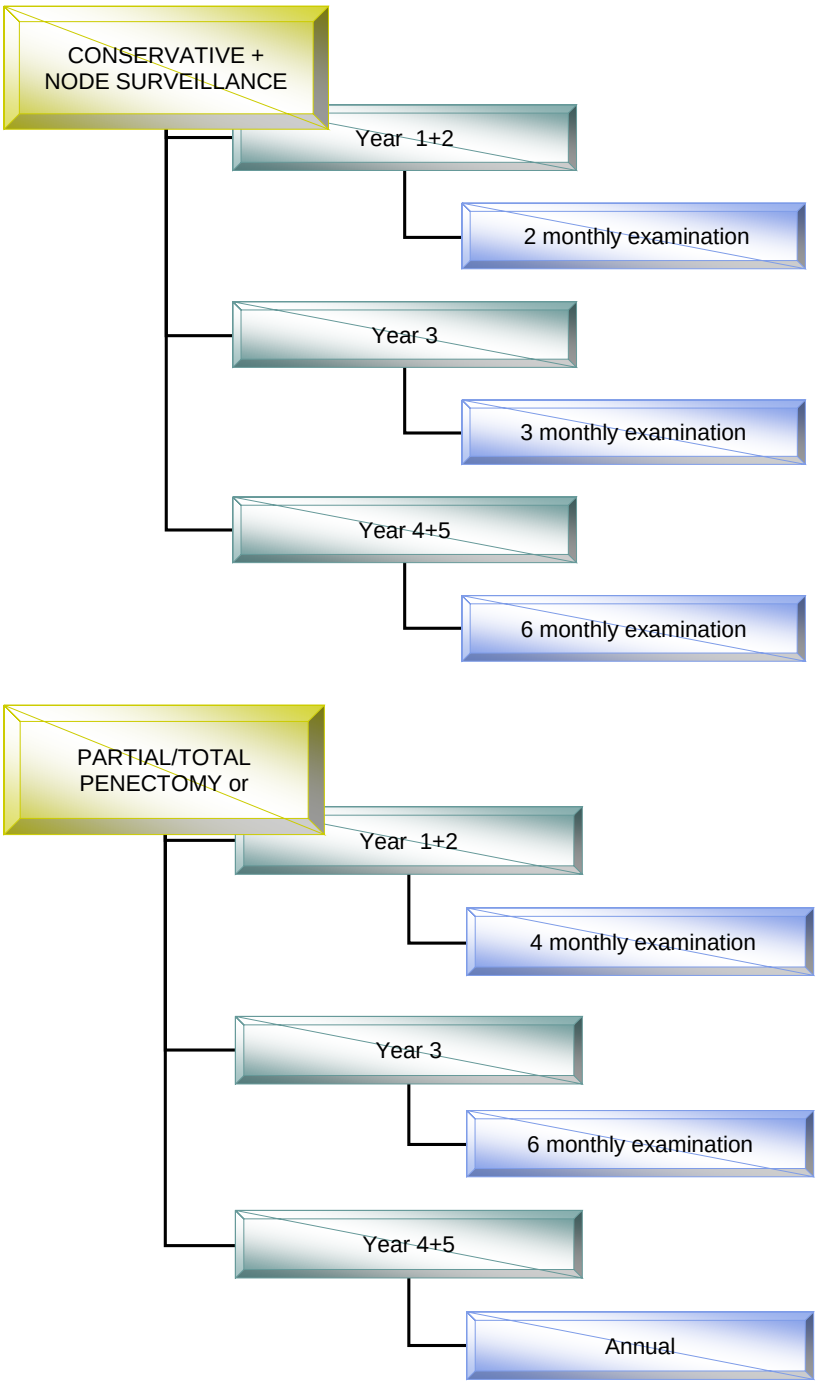
NAME

DATE	

OUTPATIENTS **NAME**

DATE	

FOLLOW UP PROTOCOL



EAU follow up protocol (modified from Algaba et al 2002)

APPENDIX D

Kayes *et al. 2009.* DNA replication licensing factors and aneuploidy are linked to tumor cell cycle state and clinical outcome in penile carcinoma. *Clin Cancer Res.* Dec 1;15(23):7335-44

Kayes *et al. 2007.* Molecular biology of penile cancer.

Lancet Oncology 2007 May;8(5):420-9